NOTCH SIGNALING IS CRITICAL FOR THE DEVELOPMENT AND SURVIVAL OF MAMMALIAN AUDITORY SUPPORTING CELLS

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ABSTRACT

The auditory sensory epithelium is critical for our ability to detect sound, and is composed of mechano-sensory hair cells and highly specialized glial-like supporting cells. Supporting cells provide structural and functional support to hair cells and play an essential role in cochlear development, homeostasis and repair. Despite their importance, little is known about the molecular mechanisms guiding supporting cell development. Previous studies revealed that the evolutionary conserved Notch signaling pathway plays an important role in the formation and maintenance of the common pool of hair cell and supporting cell progenitors (pro-sensory cells) while later during differentiation, Notch signaling becomes highly activated in a subset of pro-sensory cells destined to become supporting cells, inhibiting these cells from acquiring a hair cell fate. For the first time we provide evidence to support an instructive role for Notch signaling in supporting cell development. Using an unbiased genome wide approach we identified genes positively regulated by Notch signaling in the developing cochlea. We used genetic strategies to show that Notch signaling is both necessary and sufficient for the expression of the majority of these genes. We used two different genetic mouse models to disrupt canonical Notch signaling in differentiating supporting cells and found that Notch signaling is critical for the survival of supporting cells. Additionally we showed that a reduction in canonical Notch signaling results in defects in the proper innervation of the cochlea. Finally, we provide evidence that the different roles of canonical Notch signaling during supporting cell development are mediated by different Notch signaling components.

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CHAPTER 1

Introduction to the mammalian inner ear and the Notch signaling pathway

"All our knowledge begins with the senses, proceeds then to the understanding, and ends with reason. There is nothing higher than reason." –Immanuel Kant

An organism's ability to sense and respond to its surroundings is critical for survival. From single cell's chemotaxis response to the complexities of vertebrate vision, many types of sensory perception have evolved to meet the diverse needs of organisms to sense their environment so that they may find nourishment, select a mate to propagate the species, or avoid life-threatening danger. Vertebrate sensory systems are complex and have evolved to become highly specialized for its unique sensory modality. In general a sensory system consists of the sensory receptor cells that detect the sensory input and the neural pathway for transmitting sensory information from the receptor to the correct brain region in the sensory cortex where this information is processed and interpreted. The main sensory systems are the olfactory system, gustatory system, auditory and vestibular system, visual system, and somatic sensation system.

The auditory system is responsible for the perception of sound. Perception of sound is important for understanding spoken language and detecting potential dangers in our immediate surroundings. Our ability to hear has enabled us to develop complex spoken languages for communication as well as enhanced our culture by enabling us to perceive music and the performing arts. Unfortunately hearing loss is a common problem. Unlike lower vertebrates that can regenerate mechano-sensory hair cells after ototoxic stress or acoustic trauma, mammalian auditory hair cells are generated once during

embryonic development and then never replaced or regenerated. This lack of regenerative capacity means that once sensory cells are lost, hearing is also lost (1).

Hearing loss is a wide spread problem throughout the population affecting 4 million children and 36 million adults in the United States (2). It is estimated that two to three out of every 1,000 children born in the United States have a hearing deficiency; more than 90% of deaf children are born to hearing parents (3). Additionally hearing loss is common in an aging population. Around 15% of adult Americans over the age of 18 report some degree of hearing loss. By 45 years of age 2% of adults have a disability due to profound hearing loss, the number increasing to 50% for those who are 75 and older (2). High frequency hearing loss is a common result of noise exposure during normal everyday activities (2). Despite the prevalence of hearing disorders, there is currently no cure for hearing loss. For this reason auditory research aimed at preventing sensory cell damage or regeneration of lost sensory cells is of utmost importance for maintaining a high quality of life for an aging population.

In order to provide regenerative therapies for lost sensory cells, we must first understand the basic molecular mechanisms that drive their development. Although the molecular details of auditory hair cell differentiation have been closely studied, far less is known about the development of the auditory supporting cells. These cells are critical to the function of the sensory epithelium and may be the key to regenerative hair cell replacement therapies. In this dissertation I describe the role that the Notch signaling pathway plays in auditory supporting cell development. Through various experimental paradigms we show that Notch signaling positively regulates the supporting cell gene expression program. Using two genetic approaches we show that canonical Notch

signaling is important for the survival of a subset of supporting cells. Moreover we show that reduction of canonical Notch signaling results in innervation defects in the outer hair cell region. Finally we explore how different components of the Notch signaling pathway mediate different roles for Notch signaling in supporting cell development.

The structure and organization of the peripheral mammalian auditory system

The mammalian auditory periphery consists of the outer ear, the middle ear, and the inner ear. Sound waves enter the pinna of the outer ear and travel through the auditory canal. They then cause the vibration of the tympanic membrane commonly referred to as the eardrum. This then causes the movement of the three bones in the middle ear. The last bone in the middle ear transmits the mechanical energy to the inner ear cochlea, a fluid filled structure that contains the auditory sensory epithelium, which contains sensory cells that mediate mechano-reception. These cells are responsible for converting the mechanical stimulus produced by sound into an electrical potential that will be transmitted from the spiral ganglion to the auditory cortex of the brain.

The auditory sensory epithelium, also referred to as organ of Corti, is comprised of mechano-sensory hair cells and their interdigitating supporting cells. The structure and cellular organization of the auditory sensory epithelium is invariant and critical for its function. The auditory sensory epithelium contains three rows of outer hair cells and one row of inner hair cells (Fig1.1). Hair cells are named as such due to the presence of protrusions on their apical surface, which look like hairs coming out of the top of the cell. These protrusions, called stereocilia, are thought to contain the mechanoreceptor channel

complex that is critical for the hair cells ability to transduce sensory input. Supporting cells surrounds these hair cells. These highly specialized glial-like supporting cells, which based on morphology, location, and function, are classified as border cells, inner phalangeal cells, inner and outer pillar cells and Deiters' cells are critical to proper auditory function (Fig1.1) (4).

Supporting cells maintain structural integrity and homeostasis of the auditory sensory epithelium

Supporting cells are critical for the function of the mature auditory sensory epithelium. The first key role supporting cells play is to maintain structural integrity of the sensory epithelium. Supporting cells are enriched with cytoskeletal elements to make them sturdy so they can ensure that the sensory epithelium can withstand the mechanical stimulation produced by sound (6, 7). Furthermore the supporting cells are responsible for the characteristic structure of the sensory epithelium; specifically the pillar cells form the ridged triangular shaped tunnel of Corti that separates the inner and outer hair cell domain (Fig1.1) (5). The supporting cells span from the basal lamina to the apical lumen, whereas hair cells only have contact with the apical lumen and the supporting cells. The supporting cells' contact with the basal lamina ensures that the sensory epithelium is anchored in position. The supporting cells also anchor the hair cells to the sensory epithelium. Furthermore supporting cells express cellular junctions that form a barrier at the apical surface of the epithelium to compartmentalize fluids containing different ion concentrations (6, 7). In addition to this role in structural support, supporting cells are

critical for small molecule and ion homeostasis in the sensory epithelium. During signal transmission the hair cell releases glutamate. Supporting cells around the inner hair cell express a glutamate transporter, GLAST, which is responsible for clearing the glutamate after its release so that synaptic function can be preserved (6, 7). This function is critical because an accumulation of excess glutamate would cause excitotoxicity and damage the cellular components involved in signal transduction. Supporting cells are also thought to be involved in the recycling of potassium, which is important for maintaining the endocochlear potential that is critical for hair cell depolarization (6, 7).

Supporting cells play a critical role in auditory sensory epithelium development

Supporting cells are critical for the formation of the mosaic checkerboard pattern arrangement of hair cells and supporting cell. Supporting cells express Nectin-3, a cell adhesion molecule, that interacts with Nectin-1 on adjacent hair cells to create an alternating pattern of the two cell types (6). Another developmentally important function for the supporting cells is secreting glycoproteins and collagen that form the tectorial membrane (7). Additionally supporting cells are important for the development of proper planar cell polarity of hair cells and their stereocilia bundles. Supporting cells have localized expression of various proteins that are critical for establishing planar cell polarity of the hair cell bundles (5).

In addition to these important roles in auditory sensory epithelial development, supporting cell express neurotrophic factors that are critical for development, positional location and synaptogenesis of the auditory spiral ganglion. Secretion of NT-3 and BDNF

are two critical molecules secreted by supporting cells that are important for spiral ganglion development (6, 7). Supporting cells also express Slitrk6, a transmembrane protein that is involved with sensory neuron survival and regulation of the trophic factors NT-3 and BDNF (8). Thus without supporting cell involvement, the development of innervation would be perturbed.

Supporting cells may be the key to hair cell regeneration

The ability to regenerate new hair cells after damage has been lost in the mammalian lineage. Researchers consider supporting cells to be a potential source for regenerating new hair cells (9). In the avian system, the supporting cells play a critical role in the natural regenerative process (10). When damage occurs to hair cells, supporting cells respond by either directly trans-differentiating into new hair cells, or by re-entering the cell cycle to produce progenitor-like cells that will give rise to new hair cells and supporting cells (11).

Since supporting cells are key effectors in avian sensory cell regeneration, experiments have been done in the mammalian system to see if they would be able to provide a regenerative source of cells to replace damaged hair cells. Experiments culturing FACS-purified supporting cells showed that terminally differentiated supporting cells maintained the latent plasticity to form hair cells in culture (12). This finding suggested that there is a restrictive signal in the mammalian cochlea epithelium that inhibits a supporting cell-mediated regenerative response. Since Notch signaling is important for repressing the hair cell fate in developing supporting cell progenitors,

researchers tested whether blocking Notch signaling would promote hair cell formation. This was indeed the case, supporting cells did replace lost hair cells and they had similar electrophysiological properties to that of the original hair cells *in vitro* (13). Furthermore it was shown that Notch inhibition could promote limited hair cell regeneration after acoustic trauma *in vivo* (14). Subsequently it was shown that inhibition of Notch signaling activates Wnt singling and enables mitotic production of new hair cells from $Lgr5^{+}$ supporting cells; this result suggests that Notch signaling inhibits Wnt activation, which in turn limits regeneration (15). Taken together these experiments describe a potential role for supporting cells in the future development of hair cell replacement therapies.

Notch signaling and the development of the auditory sensory epithelium

The Notch signaling pathway is a highly conserved developmentally important pathway that was first described in *Drosophila melanogaster (16)*. This pathway is a cell contact dependent pathway in which the Notch ligand on the signaling cell activates the Notch receptor on an adjacent cell. Once activated the internal portion of the Notch receptor translocates to the nucleus where it is integrated into a transcriptional activating complex. The Notch signaling pathway controls the development of many cell types and organ systems, some examples being cardiovascular development, nervous system development, pancreatic development, and bone development (17-19). Notch signaling is also critical for multiple aspects of inner ear development (20).

Mammalian inner ear development begins with the formation of the otic placode from the thickening of ectoderm (21). The majority of the components of the inner ear develop from this otic placode. Notch signaling in combination with Wnt signaling specifies the size of the otic placode (22). Once the otic placode is specified, it invaginates into the mesenchyme to form the otic cup, which then pinches off to form the otic vesicle (23). As the otic vesicle closes, the neuroblasts that will give rise to the auditory and vestibular ganglion start to delaminate from the ventral region of the otocyst. Notch signaling is involved in determining which cells maintain the epithelial fate and form sensory cells and which are diverted to the neuronal lineage to form the auditory and vestibular ganglion (24).

Within the developing cochlea sensory progenitors are localized in the pro-sensory domain, which is a $SOX2^+$ population of cells in the otocyst flanked by a *Bmp4* and *Jag1* expressing domain. SOX2 is a high mobility group (HMG) transcription factor that is both necessary and sufficient for sensory progenitor cell specification (25). In the murine cochlea sensory progenitors are specified by embryonic day E12; JAGGED1, a Notch ligand, activates Notch singling which in turn positively regulate SOX2 in these cells through a process termed lateral induction (26). It is possible that other signaling pathways work cooperatively to specify and maintain sensory progenitors because loss of *Jag1* or *Rbpj* (the gene that encodes the transcription factor that activates Notch induced transcription) does not yield a complete loss of $SOX2^+$ sensory progenitors (27).

$SOX2^+$ sensory progenitors undergo a period of proliferation, which is followed by cell cycle exit mediated by the cell cycle inhibitor p27/KIP1 (Fig1.2) (28). Cell cycle

exit occurs in a gradient initiated in the apex and proceeds towards the base (28). After sensory progenitors become post-mitotic, differentiation of hair cells and supporting cells being in a reverse gradient (Fig 1.2). Sonic hedgehog (SHH) signaling and Notch signaling cooperate to repress differentiation in sensory progenitor cells (29). SHH is expressed in the spiral ganglion and becomes down regulated in the basal region just prior to hair cell differentiation. Progenitor cells located in the base receive less SHH and are thus released from this inhibition. A subset of these basal sensory progenitor cells up-regulate the transcription factor ATOH1. ATOH1, a basic helix loop helix (bHLH) transcription factor, is both necessary and sufficient to dive the progenitor cells to a hair cell fate (30).

Supporting cell differentiation begins shortly after hair cell differentiation. The exact mechanisms that drive supporting cell differentiation are unknown but it has been observed that newly differentiated hair cells are required for the formation of supporting cells. When hair cells fail to develop the subsequent formation of supporting cells is also disrupted (31). Additionally ectopic hair cell formation induces supporting cell-like cells adjacent to the ectopic hair cells (32). Taken together these observations suggest that a cue from newly differentiated hair cells guides supporting cell differentiation. A clear example of this is seen in pillar cell differentiation; FGF8 secreted by inner hair cells has been shown to induce the pillar cell fate in nearby sensory progenitor cells (33). The instructive signal inducing other supporting cell subtypes is currently unknown. Notch signaling is highly activated in sensory progenitors that are fated to become supporting cells making this pathway a candidate for instructing supporting cell differentiation (34).

During differentiation of hair cells and supporting cells the newly differentiated hair cells use the Notch ligands JAG2 and DLL1 to activate Notch signaling in adjacent sensory progenitor cells (Fig1.3) (35). Notch activation results in the up-regulation of repressive transcription factors (*Hes1, Hes5, Hey1, Hey2, HeyL*) that prevent the up-regulation of *Atoh1* and subsequently restrict the cell from the hair cell fate in a process termed lateral inhibition (36). Mutations in either the Notch1 receptor or in the hair cell specific Notch ligands, *Jag2* and *Dll1*, result in the over production of hair cells at the expense of supporting cells. Similarly deletions of the Notch target genes, the Hes and Hey factors, result in ectopic production of hair cells, further providing evidence for Notch signaling's hair cell repressive function (37). To date, additional roles for Notch signaling have not been examined in the developing cochlea. One of the main reasons these roles remain unexplored is because cell fate conversion in Notch inhibition models vastly reduces supporting cell numbers, leaving very few supporting cells to analyze.

Research focus: Does Notch signaling play an instructive role in supporting cell development?

It is currently unknown if Notch signaling plays an instructive role in supporting cell development. In order to address if Notch signaling has an additional, instructive role in supporting cell development we stage specifically activated or inhibited canonical Notch signaling pathway in the differentiating cochlea. Using an unbiased genome wide approach we identified genes with important developmental functions positively regulated by Notch signaling in the developing cochlea. We used genetic strategies to show that Notch signaling is both necessary and sufficient for the expression of the

majority of these genes. We used two models to disrupt canonical Notch signaling in the developing cochlea and showed that Notch signaling is critical for the survival of supporting cells. Additionally we showed that a reduction in canonical Notch signaling results in defects in the proper innervation of the cochlea. Finally we analyzed the function of two different Notch signaling components, Jag1 and Notch1, to show that differential components contribute to the different roles canonical Notch signaling is playing in supporting cell development. Our work provides novel insight into supporting cell development and highlights multiple roles for canonical Notch signaling in supporting cell development.

Figure 1.1: Schematic of the auditory sensory epithelium.

The auditory sensory epithelium is comprised of mechano-sensory hair cells and glial like supporting cells. The border cell (red) and inner phalangeal cell (red) surround the inner hair cell (blue). The inner and outer pillar cells (yellow), which comprise the tunnel of Corti, separate the inner and outer hair cell domain. The three outer hair cells (blue) are situated on top of the Deiters' cells (green).

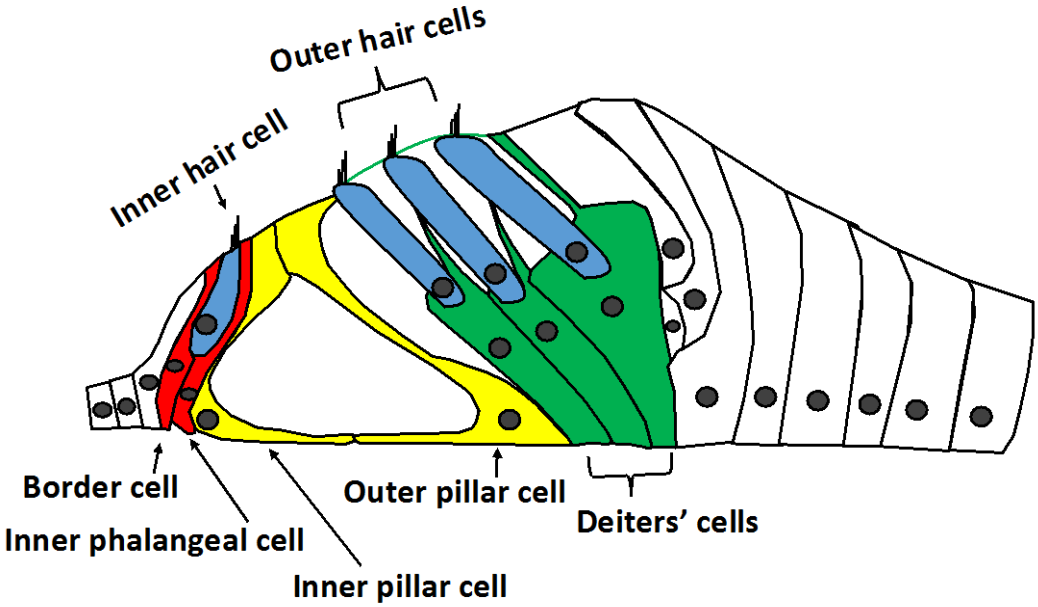

Outer hair cells
Inner hair cell
Border cell
Inner phalangeal cell
Inner pillar cell
Outer pillar cell
Deiters' cells

Figure 1.2: Schematic of sensory cell development in the cochlea.

Hair cells and supporting cells are both derived from a common pool of SOX2+ progenitor cells (1st panel). These progenitors undergo a period of proliferation and then exit the cell cycle by up-regulating, p27, a cell cycle inhibitor. Cell cycle exit occurs from the apex to the base (2nd panel). Hair cell and supporting cell differentiation occurs in a gradient from the base to the apex. Hair cells first differentiate when a subset of progenitor cells up-regulates the transcription factor ATOH1 (3rd Panel). Supporting cell differentiation shortly follows hair cell differentiation, driven by an unknown mechanism (4th panel).

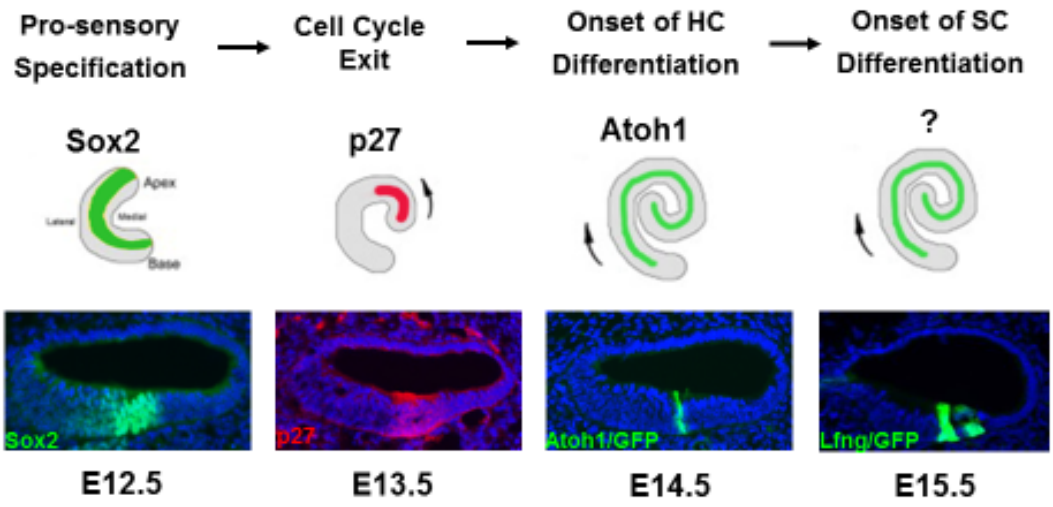
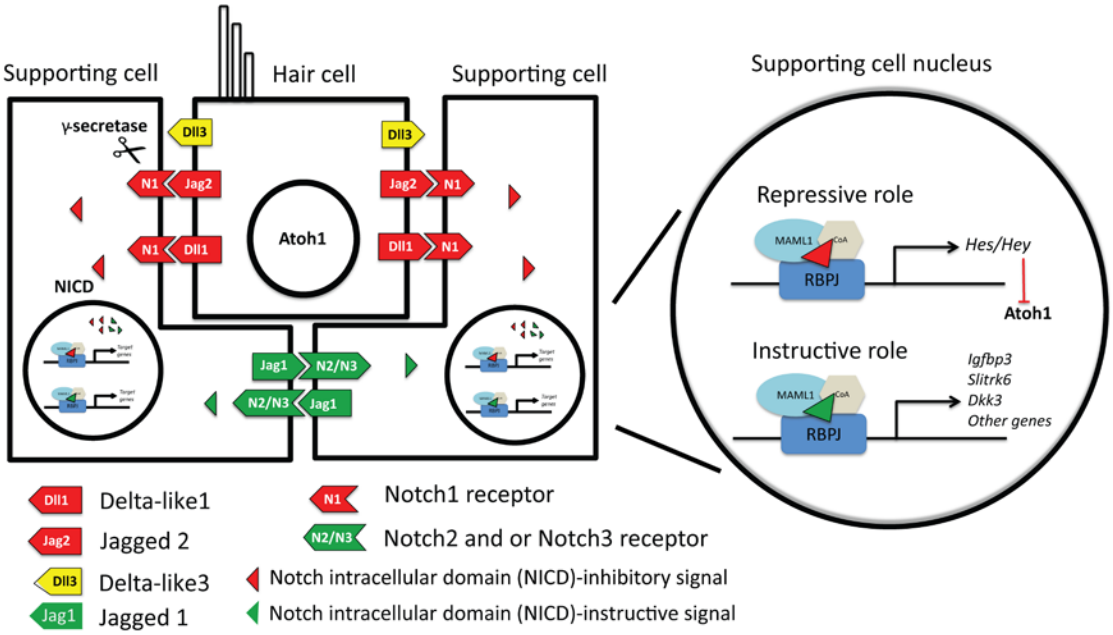

Pro-sensory Specification
Cell Cycle Exit
Onset of HC Differentiation
Onset of SC Differentiation
Sox2
p27
Atoh1
?
Apex
Medial
Lateral
Base
Sox2
p27
Atoh1/GFP
Lfng/GFP
E12.5
E13.5
E14.5
E15.5

Figure 1.3. The Notch signaling pathway in supporting cell development.

The Notch ligands Delta-like1 (Dll1) and Jagged 2 (Jag2) are expressed by newly developed hair cells. These ligands activate the Notch1 receptor on neighboring sensory progenitor cells; Notch activation induces the Hes and Hey transcription factors that repress the hair cell fate in these progenitors. In addition to this inhibitory role, Notch signaling may play an active role in supporting cell development by positively regulating supporting cell specific genes. Supporting cells also express the Notch ligand Jagged 1 (Jag1) and the Notch receptors, Notch2 and Notch3. We hypothesize that Notch ligands that bind to and signal through Notch1 promote hair cell inhibition and Notch ligands signaling through Notch2 and Notch3 are important for the instructive role of Notch signaling.


Supporting cell
Hair cell
Supporting cell
γ-secretase
Dll3
Dll3
N1
Jag2
Jag2
N1
N1
Dll1
Atoh1
Dll1
N1
NICD
Jag1
N2/N3
N2/N3
Jag1
Supporting cell nucleus
Repressive role
MAML1
CoA
RBPJ
Hes/Hey
Atoh1
Instructive role
MAML1
CoA
RBPJ
Igfbp3
Slitrk6
Dkk3
Other genes
Dll1 Delta-like1
Jag2 Jagged 2
Dll3 Delta-like3
Jag1 Jagged 1
N1 Notch1 receptor
N2/N3 Notch2 and or Notch3 receptor
Notch intracellular domain (NICD)-inhibitory signal
Notch intracellular domain (NICD)-instructive signal

CHAPTER 2

Identification of novel Notch-regulated genes in the developing mammalian cochlea

INTRODUCTION

Activation of Notch signaling results in the direct transcriptional activation of Notch target genes. In this chapter we identified and characterized genes positively regulated by Notch signaling in the developing cochlea. Notch ligand-receptor binding initiates two cleavage events, the first mediated by an ADAM-family protease and the second mediated by a γ-secretase. Once cleaved, the intracellular portion of the Notch receptor translocates to the nucleus of the cell. In the nucleus the Notch intracellular domain (NICD) associates with the transcription factor RBP-J and the mastermind-like (MAML) co-activator. This complex activates transcription of Notch target genes. Studies using gain and loss-of-function approaches have characterized Notch target genes in many systems. The best-characterized Notch target genes are the class E basic helix-loop-helix (bHLH) transcription factors related to the *Drosophila melanogaster Hairy* and *Enhancer of split* genes (38). In the cochlea these genes are *Hey1*, *Hey2*, *HeyL*, *Hes1* and *Hes5*. These genes mainly function as transcriptional repressors. In the cochlea these transcriptional repressors have been shown to repress the hair cell fate in supporting cell precursors (37, 39-41). The activated Notch complex may regulate additional genes depending on the cellular context. Other than genes involved in pro-sensory specification (*Jag1, Sox2, Fgf20*) or hair cell repression (*Hey1*, *Hey2*, *HeyL*, *Hes1*, *Hes5*), genes activated by Notch signaling in the developing cochlea have not been identified (25, 32, 37, 39, 42-45). We used the γ-secretase inhibitor, DAPT, to acutely inhibit Notch signaling in cultured cochlea epithelium at the height of supporting cell development. DAPT has been used in many studies and is widely accepted as a tool to inhibit Notch

signaling. Acute Notch inhibition allows us to identify changes in transcription resulting from Notch disruption; we wanted to avoid analyzing secondary effects that would occur due to a prolonged Notch inhibition.

We used a whole genome exon microarray to characterize changes in gene expression due to Notch inhibition. A microarray analysis allows us to identify changes in gene expression in an unbiased way. A microarray consists of a chip spotted with DNA probes that cover all known coding exons. Labeled cDNA made from RNA collected from the experimental sample is hybridized to the chip and then quantified by detecting the label bound to the probes on the chip. This method identifies and quantifies the amount of transcript for a given gene and enables comparisons of gene expression across different samples. The microarray revealed a large list of candidate genes positively regulated by Notch signaling in the developing cochlea. We validated and characterized these genes to determine if they were specifically expressed in supporting cells and positively regulated by Notch signaling.

RESULTS

Identification of novel supporting cell-specific Notch regulated genes

To gain insights into how Notch signaling impacts supporting cell development, we decided to first characterize the genes transcriptionally regulated by the Notch signaling pathway in the differentiating cochlea. To disrupt Notch signaling at the peak of supporting cell differentiation, we cultured wild type cochlear tissue at stage E15.5 in the presence of γ- secretase inhibitor (GSI) DAPT or vehicle control DMSO (control) for 19-22 hours. At the end of the culture period, we pooled control and DAPT treated explants, enzymatically purified the cochlear duct, and extracted RNA. Control and DAPT treated RNA samples from three independent experiments were analyzed using the GeneChip® Mouse Exon ST Arrays (Fig. 2.1A). Consistent with Notch's function as a hair cell-fate repressor, hair cell-specific transcription factors *Atoh1 (46)*, *Pou4f3 (47), Nhlh1* (48) and hair cell-specific Notch ligands *Dll1(49)* and *Jag2(50)* (Fig. 2.1B, red) were among the genes, which were up-regulated in response to DAPT treatment. More than 50% of the top ranked DAPT up-regulated genes (> 1.45 fold up-regulated; p value≤ 0.07) have yet to be characterized in the inner ear. The majority of these uncharacterized genes are likely expressed in hair cells, as recently revealed for *Manic fringe (Mfng)(51),* and the *Gastrin releasing peptide* (*Grp*)(52) (Fig. 2.1B, red) (Table 2.2). The top ranked DAPT down-regulated genes (> 1.45 fold down-regulated; p value≤ 0.07) contained all the documented Notch target genes functioning in hair cell fate repression (e. g. *Hey1* (37), *HeyL* (53)) and pro-sensory specification and maintenance (e.g. *Jag1* (54), *Sox2* (55)) (Fig. 1b, blue) (Table 2.1). Interestingly, among the top ranked DAPT down-regulated

genes were also genes with known functions in cell signaling (*Cyp26b1, Igfbp3)* (56) (57), neurite outgrowth and survival (*Slitrk6*) (8) and tissue homeostasis (*Slc22a3*) (58) (Fig. 1B, blue). About 20% (7 out of 34) of the top ranked DAPT down-regulated genes have been reported to be transcriptional targets of the Notch signaling pathway in other tissues (*Gucy1a3, Gucy1b3, Inhba (59); Fabp7* (60); *Igfbp3 (61); Pdgfrb (62) and Nrarp (63)*. However, for the majority of these genes, Notch-dependent regulation has not yet been reported.

Validation of Notch regulated genes in supporting cells

We validated the observed fold changes in gene expression using quantitative polymerase chain reaction assays (qPCR) in an independent Notch inhibition experiment. For the top ranked DAPT down-regulated genes (>1.43 fold down-regulated; p value ≤ 0.07), the validation rate was more than 93% (27 out of 29 tested) (Table 2.1). Among the validated DAPT down-regulated genes were known supporting cells-specific genes, namely *Slitrk6* (8), *Igfbp3* (64), *Cyp26b1* (65), *Ntf3* (66), *Lfng* (67) and *Inhba* (68). However, for the majority of the validated DAPT down-regulated genes (21 out of 27), expression in the differentiating cochlea has not yet been characterized.

We have previously shown that the p27/GFP reporter is specifically expressed in post-mitotic pro-sensory cells and differentiating supporting cells, which allows their purification by fluorescent activated cell sorting (FACS) (12, 69). We isolated supporting cells (p27/GFP+) from control (Fig. 2.2 A) and DAPT treated p27/GFP transgenic cochlear explants stage E15.5 (Fig. 2.2 B) and analyzed gene expression using RT-qPCR.

We selected *Slitrk6, Ntf3, Igfbp3, Cyp26b1*, *Inhba, Dkk3, B3galt2, Shc3, Gpr126 and Slc22a3* to be further analyzed. *Hey1*, a well characterized pro-sensory and supporting cell-specific Notch target gene, functioned as a positive control. All genes tested including the previously uncharacterized genes *Dkk3, B3galt2, Shc3, Gpr126,* and *Scl22a3* were 2-4 fold higher expressed in FACS purified supporting cells (p27/GFP$^+$ control) than unfractionated cochlear epithelial cells (CE control) (Fig. 2.2 C), demonstrating that these genes were highly enriched in supporting cells. Moreover, similar to the known Notch target gene *Hey1*, expression of *Slitrk6, Ntf3, Igfbp3, Cyp26b1, Dkk3, Inhba, B3galt2, Shc3, Gpr126* and *Slc22a3* was significantly reduced in supporting cells purified from DAPT-treated cochlear explants (p27/GFP$^+$ DAPT) compared to supporting cells purified from control cochlear explants (p27/GFP$^+$ control) (Fig. 2.2 C), suggesting that Notch signaling positively regulates their supporting cell-specific expression.

We next performed in situ hybridization (ISH) experiments on cochlear tissue stages E15.5 - E16.5 to determine whether the newly identified Notch target genes were restricted to differentiating supporting cells. At stage E15.5 and E16.5 hair cells and supporting cells have already formed in the basal cochlear segment (base, mid-base), while in the more apical segment of the cochlea (mid-apex, apex) pro-sensory cells have yet to differentiate. Based on our ISH data as well as published expression data the newly identified Notch target genes can be grouped into two categories. The first category contains genes that are highly expressed in undifferentiated hair cell and supporting cell precursors and continue to be expressed in differentiating supporting cells as shown here for *Shc3* (Fig. 2.2 E, E') and as previously reported for *Slitrk6* (8), *Ntf3*

(66), *Cyp26b1* (65). The second category contains genes that are expressed in a basal-to-apical gradient and largely limited to differentiating supporting cells and or greater epithelial cells (GER) cells as shown here for *Lnfg* (Fig. 2.2 D, D'), *Dkk3* (Fig. 2.2 F, F') and *Daam2* (Fig. 2.2 G, G') and as previously reported for *Igfbp3* (64) and *Inhba* (68).

DISCUSSION

Despite the importance of Notch signaling for vertebrate development, only a limited number of Notch target genes have been identified and characterized in various cellular contexts. In the developing cochlea, genes functioning in pro-sensory cell maintenance and hair cell fate repression have been shown to be transcriptionally regulated /co-regulated by Notch signaling (36). Here we present microarray based transcriptional profiling of GS-dependent changes in cochlear epithelial cells. We uncovered a new cohort of supporting cell-specific genes positively regulated by the Notch signaling pathway. Our study greatly expands the repertoire of pro-sensory and supporting cell-specific genes, positively regulated by Notch signaling. Among the newly identified Notch-regulated genes are genes that play key roles in cell signaling pathways including Wnt (*Dkk3 (70), Daam2 (71)*), Igf1r (*Igfbp3(72)*), Activin (*Inhba (73)*) and retinoic acid (*Cyp26b1(74)*) signaling, revealing a previously unappreciated level of cross-talk between Notch signaling and these developmentally important signaling pathways. Also among the top Notch-regulated genes are genes that are critical for cochlear innervation *(Ntf3* and *Slitrk6*)(8, 75) as well as synaptogenesis *(Ntf3)*(76), implicating a regulatory role for Notch signaling in these processes. Most interestingly, the presence of genes that function in amino acid/ neurotransmitter transport (*Slc6a14 (77), Slc22a3(78)*) and nitric oxide/cGMP signaling (*Gucy1a3, Gucy1b3*), suggests a regulatory role for Notch signaling in supporting cell physiology and cochlear homeostasis. For the first time, we show that Notch signaling is important for regulating supporting cell specific genes. Notch activation of the majority of supporting cells

specific genes has been previously unappreciated. This greater understanding of supporting cell gene regulation implicates Notch signaling in an instructive role for supporting cell development.

MATERIALS AND METHODS

Mouse breeding and genotyping:

All experiments and procedures were approved by the Johns Hopkins University Institutional Animal Care and Use Committees protocol, and all experiments and procedures adhered to National Institutes of Health-approved standards. The P27-GFP BAC transgenic line was obtained from Neil Segil (USC, Los Angeles, USA) (69). Mice were genotyped by PCR as previously described for each line. Mice of both sexes were used in this study. All mouse lines were maintained on a mixed background of C57BL/6 and CD-1.

Organotypic cochlear culture:

Cochleae from stage E15.5 P27/GFP transgenic embryos were harvested in 1x HBSS (Corning Cellgro). Cochlear tissue was enzymatically treated (see cochlear epithelial preparation) to free the cochlear duct and its innervating spiral ganglion from surrounding tissue. The cochlear duct, attached mesenchyme, and innervating spiral ganglion was placed onto filter membranes (SPI Supplies, Structure Probe) and cultured in DMEM/F12 (Corning Cellgro) supplemented with 2.5 ng/ml EGF (Sigma), 2.2 ng/ml FGF (Sigma), 1X N2 supplement (Life Technologies), 100 U/ml Penicillin (Sigma). All cultures were maintained at 37 °C in a 5% CO_2/ 20% O_2 humidified incubator. At plating half of cochlear explants cultures received DAPT (GSI) or DMSO (vehicle control). 25 mM stock solution of DAPT (*N*-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester) (Tocris Bioscience) was applied at a final concentration of 3.33

μM. Vehicle control DMSO was applied at a final concentration of 0.013 %. After culture, DMSO (control) treated cochlear explants as well as DAPT treated cochlear explants were pooled (6-10 each) and cochlear epithelia isolated. The RNA samples obtained from cochlear epithelial cells were used in microarray experiments as well as for the *in vitro* RT-qPCR based validation experiments.

Cochlear epithelial preparations:

To obtain pure cochlear epithelia, cochlear tissue was washed in CMF-PBS and incubated in dispase (1 mg/ml; Life Technologies) and collagenase (1 mg/ml; Worthington) for 8 minutes. After a 30 minute incubation in 10% FBS in DMEM-F12, non-epithelial surrounding tissue was removed by manual dissection with 30-gauge needles. For stages E17.5 and older, the cochlear capsule and the spiral ganglion were removed prior to dispase/collagenase treatment.

Cell sorting:

FACS based purification of p27/GFP+ cells was carried out on a MOFLO cytometer (DAKO-Cytomation), with a 100-μm CytoNozzle by trained staff at the Johns Hopkins Bloomberg School of Public Health Cell Sorting Core Facility. Cell dissociation and FACS based cell sorting was performed as previously described (69).

Cell sorting protocol

1. Collect GFP transgenic inner ears in HANKS buffer
    a. For embryonic inner ear (up to E17): Rinse in CMF-PBS. Add 1 mg/mL dispase + 1mg/mL collagenase in tissue culture grade CMF-PBS. Incubate

for 8 minutes. Stop reaction with 5-10% heat inactivated FBS in DMEM/F12. After 20 minutes use 27-gauge needles to dissect out cochlear duct.

b. For postnatal tissue: Gently dissect out the cochlear duct from the inner ear. Rinse in CMF-PBS. Add 1 mg/mL dispase + 1mg/mL collagenase in tissue culture grade CMF-PBS. Incubate for 8 minutes. Stop reaction with 5-10% heat inactivated FBS in DMEM/F12. After 20 minutes use 27-gauge needles to tease off the epithelial layer from the underlying mesenchyme.

2. Following collection, rinse tissue 2x in CMF-PBS and transfer cochlea to Eppendorf tube
3. Spin for 2 minutes at 1500 RPM at 4°C to collect tissue at the bottom of tube
4. Dilute 2.5% trypsin (without EDTA) to 0.05% (1:50)
    - 10µl 2.5% trypsin in 490µl CMF-PBS
5. Gently remove PBS from Eppendorf tube and replace with 300µl 0.05% trypsin. Incubate in 37°C water bath 8-10 minutes. Stop reaction with 1.2 mL of 5% FBS in DMEM/F12
6. Spin for 4 minutes at 1500 RPM at 4°C to collect tissue at the bottom of tube. Remove liquid and resuspend in 300µl of 5% FBS in DMEM/F12. Triturate a volume of 150µl rapidly and continuously for 3 minutes. Following trituration, keep dissociated cells on ice at all times! Bring volume up to 700µl for sorting.
7. Collect cells in Eppendorf tubes with 700µl of %5 serum in DMEM/F12

8. Bring cells back on ice and spin down for 20 minutes at 1500 RPM at 4°C to pellet cells. Resuspend in 50µl of DMEM/F12. Remove 10% (5µl) for quality control analysis, lyse the remaining cells in appropriate buffer for RNA extraction.
9. Quality control analysis: Add 5µl (10%) of sorted cells to 20µl DMEM/F12.
    a. Take 10µl diluted cells and add 10µl of Trypan Blue. Count using hemocytometer and calculate total number of sorted cells.
    b. Use remaining 15µl of cells for GFP analysis. Drop onto poly-d-lysine coated coverslip. Allow cells to adhere for 15min in 37°C incubator. Fix and DAPI stain. Count % GFP cells.

RNA isolation, microarray and RT-qPCR experiments:

Total RNA was extracted using the RNeasy Micro Kit (QIAGEN). For qPCR based validation experiments mRNA was reverse transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad). SYBR Green based qPCR was performed using Fast SYBR® Green Master Mix reagent (Applied Biosystems, Life Technologies #4385612) and gene-specific primers (table 3). Relative gene expression was analyzed using the ΔΔCT method (79). The ribosomal gene *Rpl19* was used as an endogenous reference gene and wild type early postnatal cochlear tissue was used as calibrator. Microarray experiments were performed at Johns Hopkins Deep Sequencing & Microarray Core Facility by trained staff. Microarray experiments were performed on three biological replicate RNA samples per condition. Total RNA was labeled using Ambion® Expression WT kit (Life Technologies). Labeled RNA was hybridized onto GeneChip®

Mouse Exon 1.0 ST Arrays (Affymetrix) and chips were scanned and analyzed according to manufactures manuals. GeneChip Expression Affymetrix CEL files were extracted and their data normalized with the Partek GS 6.6 platform (Partek Inc.). Partek's extended meta-probe set was used with RMA normalization to create quantile-normalized log2 transcript signal values, which were used in subsequent ANOVA analyses. The microarray data is deposited in the Gene Expression Omnibus (GEO) database, accession number GSE67085. Transcripts with less than a total of 12 probes as well as minor transcripts, miss-aligned and unassigned transcripts were excluded from further analysis.

RT-qPCR protocol:

cDNA preparation set up

	Master Mix 1X (using Bio-Rad iScript)
5X RT buffer*	4 µl
RT enzyme	1µl
ddH20	(20-x) µl
RNA	x µl (75ng minimum; 600-800ng ideal; 1µg maximum)

*Buffer contains oligo dT and random hexamers to prevent any 5' and 3' bias

Setting up qPCR plate

1. Primer mix: In a clean microcentrifuge tube add 6µl of each primer (100µM) (reverse and forward) and 188µl of ddH_20: 6µl+6µl+188µl=200µl (working stock solution of 3µM). Store this at -20c.

2. Make sample master mix and negative control master mix

	Master mix (1X)
ddH20	10-(5+x) µl

SYBR green 5µl

cDNA x µl (10ng minimum; 20-25ng ideal)

Add 9µl of master mix per well

Negative control master mix (1X)

ddH20 4µl

SYBR green 5µl

cDNA -

Add 9µl of master mix per well

3. Add 1µl of corresponding primer stock solution in each well followed by 9µl of sample master mix (working primer concentration is 0.3µM).

For negative control, add 1µl corresponding primer stock solution followed by 9µl of negative control solution (without cDNA).

4. Set up qPCR machine and run.

Table of primers for qPCR

Gene	Forward Primer Sequence	Reverse Primer Sequence
Abcc9	TGG AGG TCA GGA CGG ACT ATC T	GCC ACT AAT GGA TGC AAT GGA
Atoh1	ATG CAC GGG CTG AAC CA	TCG TTG TTG AAG GAC GGG ATA
B3galt2	GCA CCG AAC AGA AAC AAA GAC A	TAG CGC TCA CTT GGG TAA AGG
Chst1	GGCTACAAGATGGCCAACTCA	ACGCTCCTCCACTAGGCTGAT
Colgalt2	TTC AGT CCA AAT GCC CAG TTC	CCA TGT TGC CAC ACC AGT GT
Crhbp	TGG AGC TGC TGG GAG GAA	CAG GTC TGC TAA GGG CAT CAT C
Cybrd1	AGA CTG CCA TGG ACC TGG AA	CCG GCA TGG ATG GAT TTC
Cyp26b1	TCT GCC CCT TTG CTC TTG	ACA GGG ATC CCC TTC AGC
Daam2	GCA GTG GAA GTG GAG TTG GAA	CAG GGA CGA ACT TGT CAT TGG
Dkk3	TGT GTA CAC TGC TGG CGG CG	GAG CTC TCC CTC CAC GGG CA
Fabp7	GGA AGG TGG CAA AGT GGT GAT	TGG AAA TTG ATC TCT GTG TTC TTG A
Fgf3	GAA CGG CAG CCT TGA GAA CA	CCC ACT TCC ACC GCA GTA AT
Fgfr3	GGG CTC CTT ATT GGA CTC G	GCT CCC CTC GGA ATT CTT T
Gp5	GCC TAC GAA CCT CAC ACA CAT C	AAG CTG TGG TTC CGC AAT ATG

Gpr126	GCA ACC GGA CCC TGA GAG A	GTC ATG CCA AGC AGG AAG GT
Gucy1a3	TCC CCG CTT CGC TCT TCT	CCA GGT CTC GGT CCA GCA T
Gucy1b3	ATG AAC CTG GAC GAC CTA ACA AG	AGC ATC GTG GAG AGG GAT GT
Hes1	GCT TCA GCG AGT GCA TGA AC	CGG TGT TAA CGC CCT CAC A
Hes5	GGC GGT GGA GAT GCT CAG T	GCT GCT CTA TGC TGC TGT TGA
Hey1	CAC TGC AGG AGG GAA AGG TTA T	CCC CAA ACT CCG ATA GTC CAT
Hey2	AAG CGC CCT TGT GAG GAA A	TCG CTC CCC ACG TCG AT
Heyl	GCG CAG AGG GAT CAT AGA GAA	TCG CAA TTC AGA AAG GCT ACT G
Igfbp3	AAC CTG CTC CAG GAA ACA TCA GT	GCT TTC CAC ACT CCC AGC AT
Inhba	TCA GGC ACA GCC AGG AAG A	TGA CAG GTC ACT GCC TTC CTT
Jag1	TGT GCA AAC ATC ACT TTC ACC TTT	GCA AAT GTG TTC GGT GGT AAG AC
Lfng	ACT GCA CCA TTG GCT ACA TTG T	GGC CGC TCC GGA TGA
Mmd2	TCC CGG CGC ACA AGA G	CAC AGT TTG CTG CGT GTT CA
Moxd1	TGA CAG CGT TCT GGA CTT TGG	GAA GGC ATC GGG CAT GTT
Nrarp	TCGCTGCTGCAGAACATGAC	CTCCGGCCCGAACGA
Nckap5	TCC AAC TGC CAG ATG AGA ACA CT	GGC ATA TGT ATC GTC CCA CTG A
Ntf3	CCA AGG CAA CAG CAT GGA T	AGC TTG ATG ATG AGG GAA TTG AG
Otog	CCA TCA GCT GCC CTC CAT	GTA CCA CAG AGC CAC CAA CCT T
Pdgfrb	GTCCCATCTGCCCCTGAAA	CTGTGTAGCTGAGCACTGGTGAGT
Plp1	TGC TGC GGC CAC ACT AGT T	GAA GTT GTA AGT GGC AGC AAT CA
Rgs5	GCC CCT AAA GAG GTG AAC ATT G	GAC GGT TCC ACC AGG TTC TTC
Rpl19	GGT CTG GTT GGA TCC CAA TG	CCC GGG AAT GGA CAG TCA
S100a1	TGG ATG TCC AGA AGG ATG CA	CCG TTT TCA TCC AGT TCC TTC A
Shc3	GAG AAA GCC GCC GAG TAA GAT	GCG AAC TGG AGG TTG CTC TTC
Slc1a3	AGT GCC TAT CCA GTC CAA CGA	GGC CTC TGA CAC GTT GTT GA
Slc22a3	GCT CAT CCT TAT GTT TGC TTG GT	GCG CAT GAC AAG TCC TTG GT
Slc6a14	TCT GTG TGA CTC AGG CTG GAA	CCC ATC CAG CAC AGA AGT GA
Slitrk6	CTT CCA GCT GGG CAT TTC A	TGA TTG GAT CTG ACT CTG TAA AGC A
Sox2	CCA GCG CAT GGA CAG CTA	GCT GCT CCT GCA TCA TGC T
Tmem211	GGA AGG TCT CAG CTG CAA CAC	AAG AGC GCT GAT TGA CAG CAA
Trh	TGA TGG CTC TGG CTT TGA TCT	CAG CAA GGC GCA GGA TTT
Trhr	TCA CCG TCA CCG ATA CGT ATG T	GGC CAA GCA GGT GTC ATC A
Xist	AAG GAA ACC TGA ACA GCG TAA AA	AAT GAG ATG TGT GCA GTA AAT GCA

In Situ hybridization (ISH):

300-500bp fragments of coding sequence of *Lfng*, *Shc3, Dkk3, Daam2* cloned into pGem®-T easy (Promega, USA) were used as templates to synthesize digoxigenin-labeled antisense RNA probes according to the manufacturer's specifications (Roche

Diagnostics, GmbH). Cochlear tissue sections were rehydrated, post-fixed and incubated with proteinase K for min. Probe hybridization and washed as described by in the protocol. Bound probe was detected with anti-DIG-AP (alkaline phosphatase conjugated) antibody (Roche Diagnostics, GmbH) followed by the color reaction using the AP substrate BM Purple (Roche Diagnostics, GmbH).

Protocol for In Situ hybridization

Making probe

1) Linearizing plasmid:

-10µg Plasmid DNA

-2µl restriction enzyme

-20µl buffer

-DEPC H2O up to 20µl

2) Incubate at 37ºC at least 2 hours or o/n

3) Run 1µl linearized plasmid + 8µl DEPC H2O+ loading buffer on agarose gel for 1 hour

4) Synthesis probe (50µl):

-27.5µl dH2O

-2.5µl linear DNA

-2.5µl RNase inhibitor

-5µl DIG RNA labeled mix (final 100mM)

-10µl 5x buffer

-2.5µl RNA polymerase

5) Incubate at 37ºC for 2 hours

6) Run sample on 1% agarose gel for 30-40 minutes

-1µl RNA probe

-9µl DEPC H2O

-1µl loading buffer

7) Add 2µl DNase I to RNA probe, incubate at 37ºC for 15minutes

8) Add 100µl TE, 10µl 4M LiCl, 300ul 100% EtOH, mix and store at -20ºC (precipitates RNA)

PTw

-50ml 10x PBS

-5ml 10% Tween

-Up to 500ml of H2O

* To use RNA probe immediately after synthesis:

-Place in EtOH/dry ice bath 5 mins or till frozen

-Place in -80ºC for 2 hours

9) To use RNA probe: spin at maximum speed (13000) refrigerated (8ºC) for 30 minutes.

PK solution

-12.5ml 1x PBS

-Up to 250ml of dH2O

- 63ul of Proteinase K

10) Resuspend pellet in 40µl DEPC H2O or TE

11) Store at -20ºC

In situ hybridization on frozen sections

Day 1:

I used fresh slides (1 night to dry) so they need to bake for 20 min prior to MeOH

-15 minutes in 100% methanol at -20ºC

-Rinse in autoclaved H2O (dip 10 times)

-30 minutes air dry

-20 minutes bake slides on hot plate at 54ºC

-10 minutes 4% PFA /PBS

-Rinse in 1x PTw (1x PBS + 0.1% Tween) 10 times

0.1M TEA

-5ml TEA

-250ml dH2O

-1.250ml Acetic Anhydride

-5 minutes in PTw

-5 minutes in PTw

-Proteinase K for 3 min for E14-E16 embryonic tissue

- Rinse in PTw

- 5 minutes PTw

- 5 minutes PTw

- Rinse DEPC water

- 15 minutes Acetylation (TEA)

- Rinse PTw

- 5 minutes PTw

- 5 minutes PTw

- Rinse DEPC water

- 30 min to 1 hrs air dry slides

- Hybridize o/n at 68ºC (1ug/ml)

Hybridization buffer + probe

-20ml Hyb. buffer

-40µl probe

NTMT

-2.5M NaCl	- 20ml
-2M Tris HCl (pH9.5)	- 5ml
- 2M MgCl2	- 2.5ml
- 10% Tween 20	- 5ml
- Levamisole	- 0.24grs
- H2O	- Up to 500ml

Day 2:

- 10 minutes in 0.2X SSC at 68ºC

- 25 minutes in 0.2X SSC at 68ºC

- 25 minutes in 0.2X SSC at 68ºC

- 5 minutes in TBST at RT

- 5 minutes in TBST at RT

- 1 hour block slides in 2% Goat Serum

- 2 hours incubate in anti-DIG-AP (1/2000) in 2% TBST

- Rinse in TBST

- 5 minutes wash TBST
- 5 minutes wash TBST
- 5 minutes wash TBST
- 15 minutes in NTMT
- 15 minutes in NTMT

TBST

- 100ml 10x TBS
- 100ml 10% Tween 20
- 0.4g Levamisole
- Up to 1l H2O

- BM purple o/n for development at RT

-Stop with STOP (PtW with 0.5M EDTA 600ul in 300ml)

50ml Hybridization buffer

- 25ml Formamide
- 12.5ml 20x SSC
- 0.5ml 10%Tween 20
- 0.5ml 10% CHAPS
- 0.5ml 0.5M EDTA
- 0.5ml 100x Denhardts solution (in -20ºC freezer)
- 125ul (20mg/ml tRNA, in freezer)
- 0.005g Heparin (powder)
- Up to 50ml H2O

10x TBS (100ml)

-8g NaCl

-0.2g KCl

- 25ml 1M Tris (pH 7.5)

-75ml H2O

- Develop using the AP substrate BM Purple (Roche Diagnostics, GmbH).

Figure 2.1: Identification of Notch-regulated genes in the differentiating cochlea. (A) Schematic diagram of experimental approach used to uncover novel Notch-regulated transcripts. Transcript changes in E15.5 cochlear epithelial cells after ~20 hours of DMSO (control) or DAPT treatment were analyzed using GeneChip® Mouse Exon 1.0 ST Arrays. (B) Volcano plot of microarray data. Plotted is log2 fold-change (x-axis) versus −log10 p-value (y-axis). Data was obtained from three independent microarray experiments analyzing three independent biological replicates for control and DAPT RNA samples. Note that top ranked DAPT up-regulated transcripts are marked in dark red circles (log2 (FC) >3σ) and triangles (log2 (FC) >6σ); top ranked DAPT down-regulated transcripts are marked in dark blue circles (log2 (FC) <-3σ) and triangles (log2 (FC) <-6σ). Abbreviations: fold change (FC), standard deviation (SD).

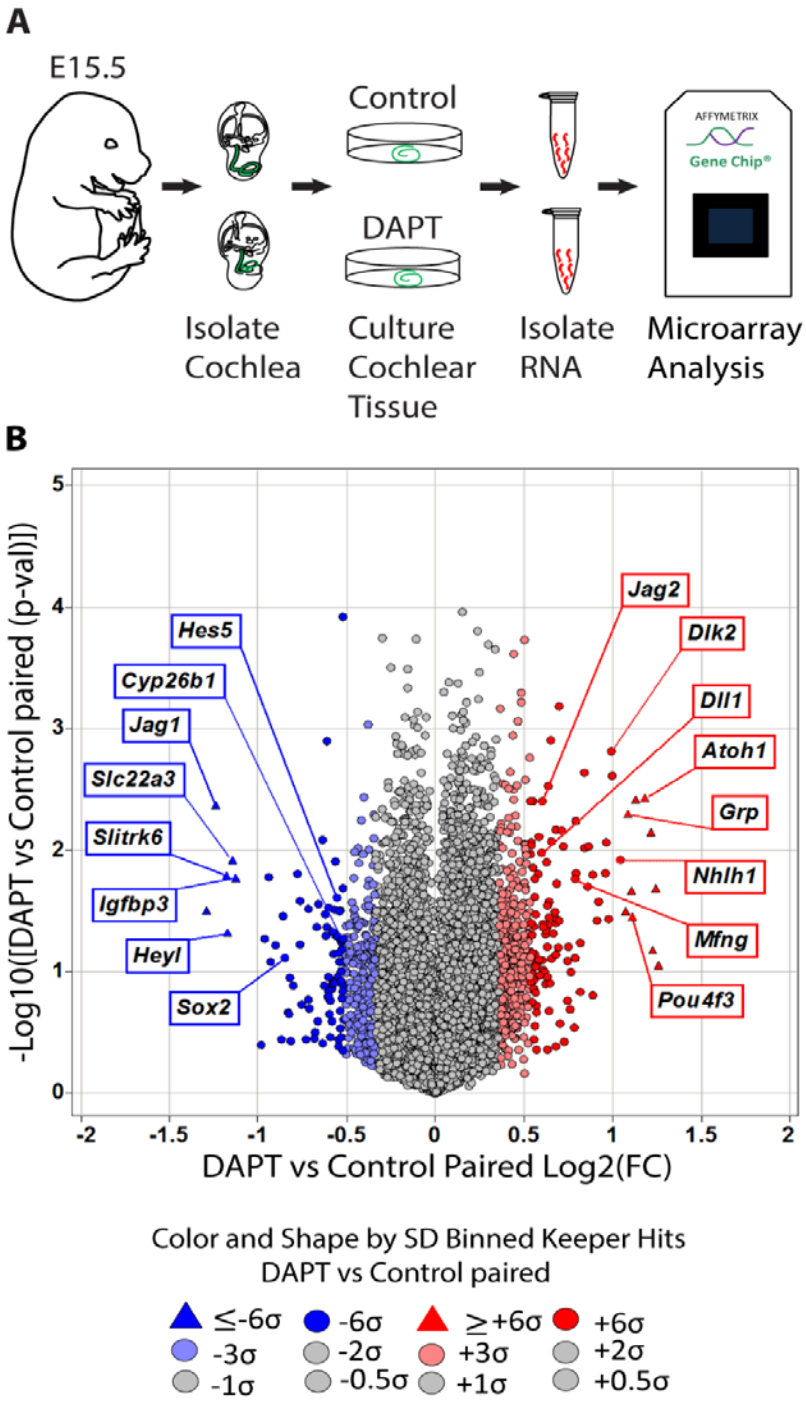

A
E15.5
Control
DAPT
AFFYMETRIX
Gene Chip®
Isolate Cochlea
Culture Cochlear Tissue
Isolate RNA
Microarray Analysis
B
-Log10([DAPT vs Control paired (p-val)])
DAPT vs Control Paired Log2(FC)
Hes5
Cyp26b1
Jag1
Slc22a3
Slitrk6
Igfbp3
Heyl
Sox2
Jag2
Dlk2
Dll1
Atoh1
Grp
Nhlh1
Mfng
Pou4f3
Color and Shape by SD Binned Keeper Hits
DAPT vs Control paired
≤-6σ
-6σ
≥+6σ
+6σ
-3σ
-2σ
+3σ
+2σ
-1σ
-0.5σ
+1σ
+0.5σ

Figure 2.2: Identification of Notch-regulated genes that are selectively expressed in supporting cells (A-C) Analysis of Notch-dependent gene expression in cochlear epithelial cells and FACS purified supporting cells. After 20 hours of culture with or without DAPT, cochlear epithelial cells (control CE; DAPT CE) obtained from E15.5 p27/GFP transgenic cochlear explants were used to FACS purify p27/GFP+ supporting cells. (A, B) FACS plots of compensated GFP fluorescence of p27/GFP transgenic CE control (A) and CE DAPT (B) cells. Blue and red box indicate gating for GFP+ supporting cells. (C) RT-qPCR was used to analyze relative expression of newly identified Notch regulated genes in CE control (black bar), CE DAPT (light grey bar), P27-GFP+ control (blue bar) and p27-GFP+ DAPT (red bar) supporting cells. Data expressed as mean ± SEM (n=3, technical replicate). (D-G) Majority of newly identified Notch-regulated genes are expressed in differentiating supporting cells and greater epithelial (GER) cells. Low and high power images (‘) of stage E15.5 (D, D’) and E16.5 (E-G’) cochlear sections labeled for *Lfng* (D, D’) *Shc3* (E, E’), *Dkk3* (F, F’) and *Daam2* (G, G’) transcript. Black line marks sensory domain. Scale bar 100μm.

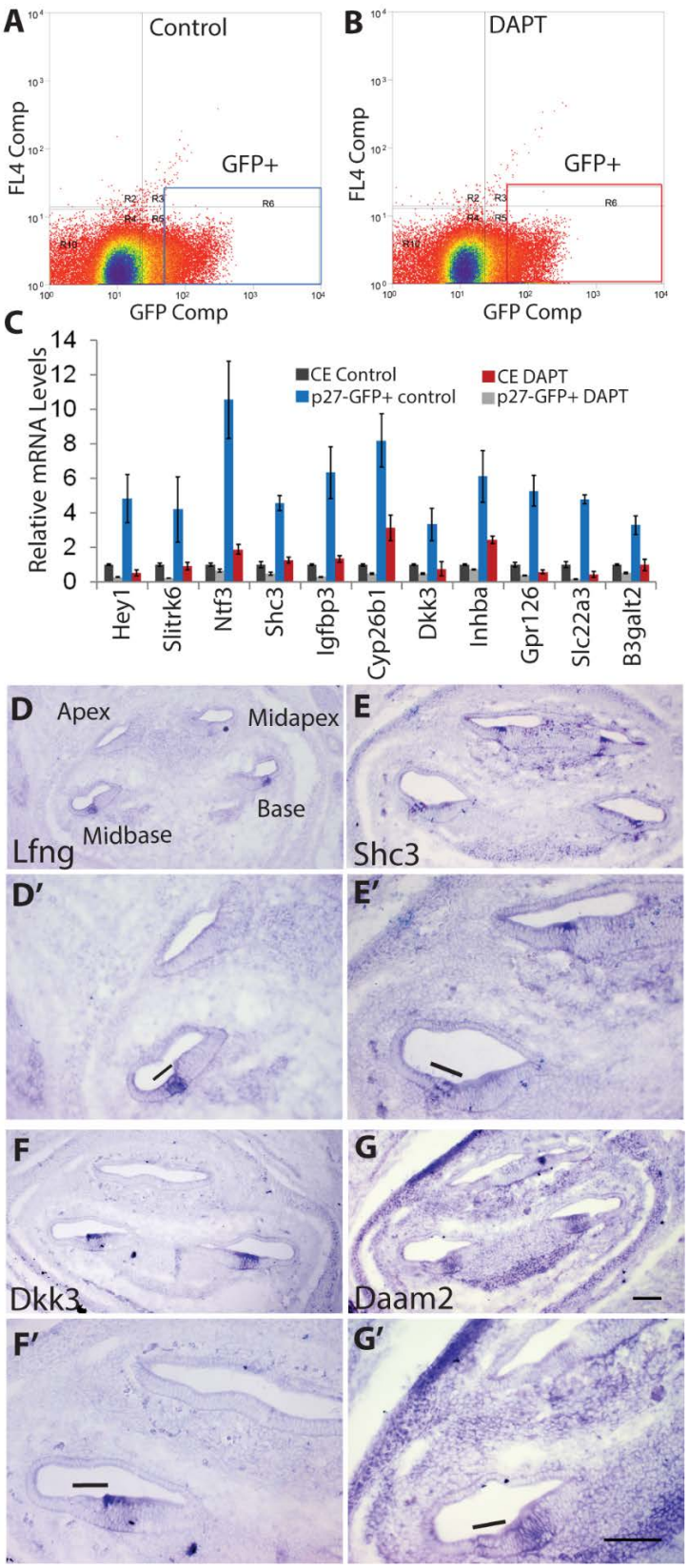

A
Control
GFP+
FL4 Comp
GFP Comp
B
DAPT
GFP+
FL4 Comp
GFP Comp
C
Relative mRNA Levels
CE Control
p27-GFP+ control
CE DAPT
p27-GFP+ DAPT
Hey1
Slitrk6
Ntf3
Shc3
Igfbp3
Cyp26b1
Dkk3
Inhba
Gpr126
Slc22a3
B3galt2
D
Apex
Midapex
Base
Midbase
Lfng
E
Shc3
D'
E'
F
Dkk3
G
Daam2
F'
G'

Table 2.1: List of genes down-regulated in response to DAPT treatment.

ND, not determined, * indicates known Notch target gene, ** not validated

SD DAPT vs Control Log2(FC)	*Gene Symbol*	*Gene AccID*	*P-value DAPT vs Control*	*Mean DAPT*	*Mean Control*	*Ratio DAPT vs Control*	*qPCR*
≤ -6σ	Hey1	NM_010423	0.007	9.23	10.52	0.41	ND*
≤ -6σ	Jag1	NM_013822	0.002	10.06	11.30	0.42	ND*
≤ -6σ	Slitrk6	NM_175499	0.002	10.83	12.01	0.44	0.17
≤ -6σ	Heyl	NM_013905	0.020	8.08	9.25	0.44	ND*
≤ -6σ	Slc22a3	NM_011395	0.012	7.76	8.91	0.45	0.13
≤ -6σ	Igfbp3	NM_008343	0.004	9.23	10.36	0.46	0.33
-6σ	Fabp7	NM_021272	0.054	8.56	9.53	0.51	0.34
-6σ	B3galt2	NM_020025	0.017	8.73	9.67	0.52	0.49
-6σ	Abcc9	NM_021041	0.060	7.45	8.35	0.53	0.28
-6σ	Fam159b	NM_029984	0.012	5.99	6.85	0.55	ND
-6σ	Sox2	NM_011443	0.021	11.22	12.07	0.55	ND*
-6σ	Shc3	NM_009167	0.020	9.56	10.38	0.57	0.45
-6σ	Hey2	NM_013904	0.016	9.38	10.16	0.58	ND*
-6σ	Hes1	NM_008235	0.009	8.49	9.25	0.59	ND*
-6σ	Trh	NM_009426	0.012	7.38	8.14	0.59	0.13
-6σ	Gucy1b3	NM_017469	0.007	7.59	8.31	0.61	0.56
-6σ	Colgalt2	NM_177756	0.016	9.09	9.76	0.63	0.41
-6σ	Inhba	NM_008380	0.003	9.32	9.98	0.63	0.35
-6σ	Crhbp	NM_198408	0.001	6.52	7.16	0.64	0.78**
-6σ	Gucy1a3	NM_021896	0.034	8.49	9.11	0.65	0.49
-6σ	Gpr126	NM_001002268	0.051	8.01	8.63	0.65	0.52
-6σ	Tmem211	NM_001033428	0.001	7.76	8.38	0.65	0.32
-6σ	Gp5	NM_008148	0.032	5.61	6.21	0.66	ND
-6σ	Dkk3	NM_015814	0.030	8.42	9.02	0.66	0.45
-6σ	Xist	NR_001463	0.046	11.40	11.99	0.67	7.04**

-6σ	Nckap5	NM_172484	0.070	7.17	7.75	0.67	0.47
-6σ	Trhr	NM_013696	0.034	6.68	7.25	0.67	0.33
-6σ	Ntf3	NM_001164034	0.042	8.99	9.57	0.67	0.31
-6σ	Cybrd1	NM_028593	0.021	9.55	10.12	0.67	0.38
-6σ	Rgs5	NM_009063	0.056	8.43	9.00	0.67	0.51
-6σ	Moxd1	NM_021509	0.050	10.50	11.06	0.68	0.62
-6σ	Mmd2	NM_175217	0.012	8.26	8.82	0.68	0.52
-6σ	Hes5	NM_010419	0.008	9.12	9.68	0.68	ND*
-6σ	Lacc1	BC116748	0.004	8.89	9.43	0.69	ND
-6σ	Fgf20	NM_030610	0.029	8.38	8.92	0.69	ND*
-6σ	Lfng	NM_008494	0.003	9.23	9.76	0.69	0.38
-6σ	Nrarp	NM_025980	0.012	9.20	9.73	0.69	0.27
-6σ	Pdgfrb	NM_001146268	0.065	9.67	10.20	0.69	0.48
-6σ	Cyp26b1	NM_175475	0.054	8.23	8.76	0.70	0.29
-6σ	C030013G03Rik	AK021075	0.021	5.07	5.60	0.70	ND
-6σ	Chst1	NM_023850	0.065	9.23	9.75	0.70	0.25

Table 2.2: List of genes up-regulated in response to DAPT treatment.

SD Binned Keeper DAPT vs Cont paired Log2(FC)	*Gene Symbol*	*Unique Gene AccID*	*DAPT vs Cont (p-val)*	*Mean paired (DAPT)*	*Mean paired (Cont)*	*DAPT vs Cont paired (ratio)*
> +6σ	Tmem173	NM_028261	0.001	10.31	9.07	2.37
> +6σ	Atoh1	NM_007500	0.003	11.54	10.32	2.32
> +6σ	Acbd7	NM_030063	0.004	7.59	6.46	2.19
> +6σ	Pou4f3	NM_138945	0.004	11.08	9.97	2.16
> +6σ	Gm6537	NM_001195091	0.008	10.43	9.33	2.16
> +6σ	Grp	NM_175012	0.004	9.26	8.17	2.12
> +6σ	Tmem255b	NM_001143671	0.030	10.40	9.33	2.11
+6σ	Nhlh1	NM_010916	0.004	8.78	7.74	2.06
+6σ	Calb2	NM_007586	0.002	8.32	7.32	2.00
+6σ	Dlk2	NM_207666	0.002	10.11	9.12	1.99
+6σ	Ush2a	NM_021408	0.037	8.28	7.30	1.97
+6σ	Chrna10	NM_001081424	0.016	8.39	7.43	1.94
+6σ	Ptprq	NM_001081432	0.038	9.08	8.17	1.88
+6σ	Kcna10	NM_001081140	0.016	6.64	5.76	1.85
+6σ	Dll3	NM_007866	0.009	9.09	8.23	1.82
+6σ	Rasd2	NM_029182	0.002	10.07	9.22	1.79
+6σ	Scn11a	NM_011887	0.010	7.88	7.04	1.79
+6σ	Lhfpl5	NM_026571	0.006	8.58	7.78	1.73
+6σ	Rbm24	NM_001081425	0.005	8.26	7.47	1.73
+6σ	Foxj1	NM_008240	0.026	9.30	8.51	1.73
+6σ	Mfng	NM_008595	0.002	9.83	9.04	1.73
+6σ	Steap4	NM_054098	0.046	6.90	6.13	1.70
+6σ	Grxcr2	NM_001033426	0.062	8.51	7.76	1.68
+6σ	Gad2	NM_008078	0.004	7.84	7.12	1.65
+6σ	Serpine3	AK053602	0.007	7.19	6.47	1.64
+6σ	Tmprss7	NM_172455	0.001	7.23	6.53	1.62
+6σ	Gfi1	NM_010278	0.058	8.48	7.79	1.62

+6σ	Rtn4rl2	NM_199223	0.007	9.92	9.24	1.60
+6σ	Lhx3	NM_001039653	0.040	8.95	8.27	1.60
+6σ	Dysfip1	NM_026814	0.048	7.53	6.86	1.59
+6σ	Mgat5b	NM_172948	0.009	9.36	8.71	1.58
+6σ	Gm88	BC147714	0.001	8.69	8.04	1.57
+6σ	Slc26a5	NM_030727	0.036	6.78	6.14	1.56
+6σ	Otof	NM_031875	0.027	8.89	8.26	1.56
+6σ	Oacyl	NM_177028	0.003	8.30	7.66	1.56
+6σ	Gimap4	NM_174990	0.041	7.19	6.55	1.55
+6σ	Tph1	NM_001136084	0.059	7.21	6.57	1.55
+6σ	Ccl3	NM_011337	0.062	8.86	8.23	1.55
+6σ	Gm2694	NR_033430	0.044	7.53	6.90	1.55
+6σ	Bsnd	NM_080458	0.046	9.02	8.40	1.54
+6σ	Thsd7b	NM_172485	0.017	7.87	7.25	1.54
+6σ	Fpr1	NM_013521	0.064	5.83	5.22	1.53
+6σ	Aqp1	NM_007472	0.040	9.56	8.95	1.52
+6σ	Jag2	NM_010588	0.004	10.15	9.55	1.52
+6σ	Dll1	NM_007865	0.000	8.01	7.41	1.52
+6σ	Ankrd22	NM_024204	0.020	8.76	8.17	1.50
+6σ	Wfikkn2	NM_181819	0.008	9.47	8.88	1.50
+6σ	Tesc	NM_021344	0.011	8.09	7.52	1.48
+6σ	Slc52a3	NM_027172	0.017	9.86	9.30	1.48
+6σ	Gm11992	NM_001037928	0.024	7.35	6.79	1.48
+6σ	Apln	NM_013912	0.048	8.78	8.22	1.47
+6σ	Mep1a	NM_008585	0.008	6.03	5.47	1.47
+6σ	Srrm4	NM_026886	0.014	7.64	7.09	1.47
+6σ	Gm8075	XR_105495	0.004	5.75	5.20	1.47
+6σ	Casz1	NM_027195	0.009	8.36	7.82	1.46
+6σ	Sstr2	NM_001042606	0.040	8.99	8.45	1.45
+6σ	Pde2a	NM_001143848	0.009	8.62	8.08	1.45
+6σ	Cbln1	NM_019626	0.004	7.58	7.05	1.45
+6σ	1700003M02Rik	NM_027041	0.068	7.24	6.70	1.45
+6σ	Fam183b	NM_029283	0.011	6.61	6.07	1.45

CHAPTER 3

Notch signaling is necessary and sufficient for cochlear expression of the newly identified Notch target genes *in vivo*

INTRODUCTION

Notch signaling has been extensively studied for its role in repressing the hair cell fate in supporting cell precursors but an instructive role for Notch in supporting cell differentiation has not yet been described. Using a GSI to inhibit the Notch pathway followed by a microarray analysis, we identified a new set of genes positively regulated by Notch signaling in the developing cochlea. This finding suggests that Notch signaling is important for the regulation of many supporting cell specific genes that may be important for development and/or function of supporting cells. Although GSIs like DAPT are widely used to inhibit the Notch signaling pathway, some of the observed changes in gene expression may be due to the inhibition of GS-dependent processes other than Notch signaling (80). In this chapter we will use an *in vivo* gain and loss-of-function approach to validate the Notch regulation of these newly identified genes.

Previous Notch loss-of-function studies focused on the role of Notch signaling in pro-sensory specification or the role of Notch signaling lateral inhibition of the hair cell fate (36). The *Jag1* and *Rbpj* mutants revealed Notch signaling's involvement in maintenance of the sensory progenitors (26, 27, 81). In these mutants there are reduced numbers of both hair cells and supporting cells presumable due to cell death (27, 82). Although there is not complete loss of $SOX2^{+}$ sensory progenitors, the severe reduction of sensory cells led to the conclusion that Notch at this early stage is critical for maintaining sensory progenitors. During differentiation of hair cells and supporting cells Notch signaling has been shown to repress the hair cell fate in supporting cell precursors. Evidence for this role comes from the *Notch1* mutant in which hair cells are over produced at the expense of supporting cells. Mutants of the hair cell-specific Notch

ligands, *Dll1* and *Jag2,* also have an ectopic hair cell phenotype, further showing that Notch signaling is involved in lateral inhibition of the hair cell fate. (83). One limitation to these mutant models is that loss of supporting cells due to conversion makes it difficult to analyze the resulting supporting cell phenotype. Thus we wanted to select a new model to disrupt canonical Notch signaling in the inner ear that would allow us to retain some supporting cells for analysis. For our loss-of-function model we decided to use a recently developed Notch hypomorphic mouse model, the DnMAML1 mutant mouse line.

The DnMAML1 mouse line is a knock-in into the *Rosa26* locus of a floxed stop cassette followed by the sequence for a truncated form of the human MAML1 gene fused to GFP. Upon Cre-mediated excision of a stop cassette, the *Rosa26* promoter drives the expression of a truncated form of the human MAML1 protein fused to GFP (84). This fusion protein forms a complex with the intracellular domain of the Notch receptor and RBPJ, rendering a transcriptionally inactive complex, and thus blocking Notch-mediated transcriptional activation (Fig. 3.1 A). To independently confirm that canonical Notch signaling is indeed required for the regulation of the newly identified DAPT down-regulated genes, we used RT-qPCR to analyze changes in gene expression of DAPT down-regulated genes in the DnMAML1 mutant versus the control. This mouse has not been used in the inner ear, and represents a novel strategy to disrupt canonical Notch signaling in supporting cells.

Previous gain-of-function studies were focused on the ability of Notch signaling to form new sensory regions (32, 85). When the intracellular domain of Notch1 was over expressed in the developing inner ear otocyst, ectopic sensory patches, containing hair cells and supporting cells formed (32, 85-87). In the non-sensory regions of the cochlea,

Notch's ability to induce ectopic sensory patches is limited to stages prior to E13 (86). Later induction of Notch signaling in the cochlea leads to ectopic SOX2+ patches that do not contain any MYO6+ hair cells. The identity of these $SOX2^{+}$ cells has not been analyzed in detail. We reason that if Notch signaling has an instructive role in supporting cell differentiation, that these cells could be expressing supporting cell specific genes. To activate Notch signaling we used a mutant mouse model that has a floxed stop cassette inserted before the N1ICD coding sequence knocked into the *Rosa26* locus. Cre-mediated recombination removes the stop cassette and enables over-expression of N1ICD (32). We used RT-qPCR to show that activation of Notch results in activation of a supporting cell gene expression program.

RESULTS

Canonical Notch signaling controls newly identified Notch regulated genes *in vivo*.

To induce early inner ear-specific expression of DnMAML1 we used the well characterized inner ear-specific Pax2-Cre line (88). Pax2-Cre; Rosa26 $^{DnMAML1/+}$ animals were examined at E18.5 to circumvent neonatal lethality. At E18.5, hair cell and supporting cell differentiation is largely completed, and in the wild type (control) cochlea myosinVIIa (MYO7A) positive hair cells are arranged in three rows of outer hair cells and one row of inner hair cells (Fig. 3.1 B, F). In the DnMAML1 expressing cochlea, hair cells were severely miss-patterned and the number of inner hair cells was significantly increased compared to control (Fig. 3.1 C, G, H). Moreover, in contrast to the uniform orientation of actin-rich hair cell bundles seen in control cochleae, hair cell bundles were severely disoriented in the DnMAML1 cochleae (Fig. 3.1 B, C). At E18.5 supporting cells are largely differentiated and based on the morphology and location of their $SOX2^{+}$ nuclei can classified as inner border and inner phalangeal cells, inner and outer pillar cell and Deiters' cells (Fig. 3.1 D, F). Supporting cells were largely retained in DnMAML1 expressing cochlea; however Deiters' cell nuclei were enlarged and the density of mid-basally located Deiters' cells was modestly reduced in DnMAML1 expressing cochlea compared to control (Fig. 3.1 E, I).

To determine whether the newly identified DAPT down-regulated supporting cell-specific genes are positively regulated by Notch signaling *in vivo*, we isolated cochlear epithelia from E18.0 DnMAML1 mutant embryos and wild type (control) littermates, prepared RNA and performed RT-qPCR to analyze gene expression. The

known direct Notch target genes *Hes5, Hey1, Sox2* and *Jag1* served as positive controls. *S100a1*, which encodes for Ca^{2+} binding protein and is highly expressed in Deiters' cells and pillar cells served as negative control (89). As expected, we found that *Hes5, Hey1, Sox2* and *Jag1* transcripts were significantly down-regulated in the DnMAML1 expressing cochlear epithelia, whereas the expression of the supporting cell marker gene *S100a1* was not significantly reduced (Fig. 3.1 J). Furthermore, our RT-qPCR experiments revealed that all the examined genes, namely *Slitrk6, Ntf3, Igfbp3, Cyp26b1, Dkk3, Daam2, Shc3, B3galt2, Colgalt2* and *Slc22a3,* were significant down-regulated in DnMAML1-expressing cochlear epithelia as compared to wild type cochlear epithelia (Fig. 3.1 J), indicating that canonical Notch signaling pathway is required to maintain supporting cell-specific gene expression *in vivo.*

Ectopic Notch signaling activates a supporting cell-specific gene program.

We examined whether Notch over-activation a time point in which ectopic hair cells are not induced is sufficient to induce the newly identified Notch-regulated genes. Specifically, we used the $Emx2^{Cre/+}$ line (90), which turns on Cre expression at around E13.5 (37) and allows for ectopic expression of N1ICD ($Rosa26^{N1ICD}$) (91) throughout the cochlear epithelial duct.

As $Emx2^{Cre/+}$; $Rosa26^{N1ICD/+}$ (N1ICD) animals die at birth our analysis was limited to late embryonic stages (E18.0-E18.5). We found, as expected, scattered clusters of ectopic $SOX2^{+}$ cells throughout non-sensory regions of N1ICD overexpressing cochleae. Moreover, consistent with previous reports we did not observe any ectopic

MYO7A+ hair cells within these ectopic SOX2 clusters (Fig. 3.2 C, C"). Interestingly, in contrast to the normal compliment of three outer hair cells seen in wild type (control) cochleae (Fig. 3.2 A, A'), outer hair cells were frequently missing in the N1ICD over-expressing cochlea, ranging from only two outer hair cells to no outer hair cells (Fig. 3.2 C, C'). To determine whether Notch activation is sufficient to drive the expression of the newly identified Notch-regulated genes, we isolated cochlear epithelia from E18.0 N1ICD mutant embryos and wild type (control) littermates, prepared RNA and used RT-qPCR to analyze gene expression in control and N1ICD over-expressing samples. Notch target genes *HeyL* and *Jag1* functioned as positive controls. 11 out of the 12 newly identified Notch-regulated genes tested showed an increase in gene expression as a result of Notch over-activation. The expression of *Igfbp3, Slc6a14, Slitrk6, Daam2, Shc3, Dkk3, Gpr126* and *Inhba* was significantly increased in response to Notch over-activation; *Scl22a3, Ntf3* and *Cyp26b1* expression was increased, but the level of induction varied substantially across N1ICD samples (Fig. 3.2 E). We expanded our RT-qPCR based gene expression analysis to examine the expression of additional genes characteristic of supporting cells (*S100a1*(89)*, Slc1a3* (GLAST) (92), *Otog*(93), *Plp1*(94) and *Fgfr3*(95)). RT-qPCR revealed that with the exception of *Plp1*, the expression of each of the supporting cell-specific marker genes was significantly up-regulated in N1ICD over-expressing cochlear epithelial cells compared to control cochlear epithelial cells (Fig. 3.2 E). These observations suggest that Notch signaling is sufficient to activate a supporting cell-specific gene expression program in the differentiating cochlea.

To determine whether Notch over-activation produced ectopic supporting cell-like cells we stained E18.0 wild type and N1ICD over-expressing cochlear tissue with a pan

S100 antibody, which in the neonatal cochlea marks Deiters cells and pillar cells (96). In wild type (control) cochlear tissue, anti-S100 staining marked $SOX2^+$ pillar cells and $SOX2^+$ Deiters cells; outside the sensory epithelium anti-S100 staining marked $SOX2^-$ cells of the presumptive stria vascularis (Fig. 3.2 B, B')(97). In the N1ICD over-expressing cochleae, ectopic anti-S100 staining was observed in the hair cell layer atop of pillar cells and Deiters cells, suggesting that outer hair cell precursors switched fate and differentiated as supporting cell-like cells (Fig. 3.2 D, D'). We also observed infrequently clusters of $SOX2^+$ $S100^+$ cells outside the sensory epithelium, however the majority of ectopic $SOX2^+$ clusters were $S100^-$ (Fig. 3.2 D, D"). In summary, our findings indicate that Notch signaling promotes a supporting cell-specific gene expression program in the differentiating cochlea, and is sufficient to instruct a supporting cell fate in a subset of cells including outer hair cell precursors.

DISCUSSION

The molecular cues that drive auditory supporting cell development are poorly understood. Here we provide evidence that Notch signaling plays an instructive role in supporting cell development by positively regulating a subset of the supporting cell transcriptome. Using both Notch loss-of-function (DnMAML1) and Notch gain-of-function (N1ICD) models, we were able to demonstrate that Notch signaling is both necessary and sufficient for the expression of the newly identified supporting cell-specific Notch regulated genes. Moreover, we show that constitutive Notch signaling promotes a supporting cell-specific program in the differentiating cochlea and is sufficient to render outer hair cell precursors as well as a subset of non-sensory cochlear epithelial cells into supporting cell-like cells.

Is Notch signaling sufficient to ectopically induce a supporting cell fate? Our analysis of the Emx2 $^{Cre/+}$; Rosa26 $^{N1ICD/+}$ mutant cochlea suggests that Notch signaling is sufficient to induce a supporting cell-specific gene expression program in the differentiating cochlea. However, the ability of Notch signaling to induce ectopic supporting cell-like-cells within the differentiating cochlea is highly cell context dependent. The most amendable cells for Notch-mediated reprogramming appear to be outer hair cell precursors. Previous studies have shown that Notch over-activation in undifferentiated vestibular sensory epithelia results in fewer hair cells being produced (32, 54). Our data suggests that ectopic Notch activation in outer hair cell precursors not only represses a hair cell-specific program, but also instructs outer hair cell precursors to differentiate into S100^{+} and SOX2^{+} outer supporting cell-like cells. In contrast to outer

hair cells, inner hair cells appeared to form relatively normal in Emx2 $^{Cre/+}$; Rosa26 $^{N1ICD/+}$ mutant cochlea. Inner hair cells are the first cells to differentiate and it is likely that at the time of Notch activation these cells had already initiated a hair cell-specific program and were too far advanced to be reprogrammed. Support for this idea comes from two recent studies, which show that ectopic Notch activation in nascent auditory hair cells is not sufficient to derail the initial phase of their hair cell-specific program (86) (98), and only in the mature cochlea does the loss of inner and outer hair cell-specific characteristics become evident (98).

Although we activated Notch signaling throughout the entire cochlear epithelium, we only infrequently observed ectopic patches of supporting cell-like cells in non-sensory regions of the epithelium, suggesting that the ability of Notch signaling to induce a supporting cell-like fate outside the sensory epithelium is limited and highly dependent on cell context. Recent studies have shown that other genes are able to restrict sensory domain formation in non-sensory regions. One gene, *Lmo4* is expressed in the ventral cochlea duct and disruption of this gene results in the formation of an ectopic sensory region that is a mirror image to the endogenous auditory sensory region indicating that LMO4 represses sensory cell development (99). Presence of LMO4 protein may limit Notch's ability to induce supporting cell-like cells due to its antagonistic role in sensory fate induction. *Otx2* has been shown to also repress the formation of sensory regions in the non-sensory regions of the cochlea (100). Thus, like *Lmo4,* expression of *Otx2* may limit Notch's ability to form sensory cells in non-sensory regions where it is expressed. Taken together this suggests that for Notch signaling to be able to form sensory cells, or supporting cell-like cells, when ectopically activated, the cell must also be permissive for

sensory cell development. Lack of additional pro-sensory factors or the presence of sensory fate inhibitors may reduce and/or block Notch's ability to induce the supporting cell fate in non-sensory epithelial cells. Overall, our data show that Notch signaling is critical for regulating a large number of supporting cell specific genes; within a permissive context Notch can induce supporting cell-like cells, thus suggesting an instructive role for Notch signaling in supporting cell development.

MATERIALS AND METHODS

Mouse breeding and genotyping:

All experiments and procedures were approved by the Johns Hopkins University Institutional Animal Care and Use Committees protocol, and all experiments and procedures adhered to National Institutes of Health-approved standards. The Pax2-Cre BAC transgenic line was obtained from Andrew Groves (Baylor College, Houston, USA) (88). The Emx2 $^{Cre/+}$ knock in line was obtained from Shin Aizawa (RIKEN, Kobe, Japan)(90). The Cre inducible Rosa26 $^{Dn\text{-}MAML1/+}$ line was obtained from Warren Pear (Univ. of Pennsylvania, Philadelphia, USA). The Cre inducible Rosa26 $^{N1ICD/+}$ line (91) (#008159) and Ai14 Cre reporter line (101) (#007914) was purchased (Jackson Laboratory, Bar Harbor, USA). Mice were genotyped by PCR as previously described for each line. Mice of both sexes were used in this study. All mouse lines were maintained on a mixed background of C57BL/6 and CD-1.

Tissue processing and staining:

Embryonic and postnatal animals were staged using the EMAP eMouse Atlas Project (http://www.emouseatlas.org) Theiler staging criteria. To allow sectioning, whole heads (Stages E15-E17) or dissected inner ears (Stage E18 –P5) were fixed in 4% para-formaldehyde (PFA) in 1X PBS overnight, put through a sucrose gradient (10% sucrose for 30 minutes, 15% sucrose for 30 minutes and 30% sucrose overnight), submerged in OCT (Tissue-Tek, Sakura) and flash frozen. 14μM thick tissue sections were collected on SuperFrost Plus slides (Fisher). To obtain a cochlear surface preparation, PFA fixed

cochlear tissue was dissected in 1X PBS to remove cochlear capsule, the cochlear roof and the innervating spiral ganglion.

Immuno-staining: cochlear tissue was washed three times with 1X PBS 5-10 minutes each and blocked with 1X PBS containing 10% Normal Donkey Serum (Sigma) and 0.5% TritonX100 (Sigma) for 30 minutes. Immuno-staining was performed according to the manufacture's specifications. Primary antibodies: rabbit anti-myosinVIIa (1:500, Proteus #25-6790), goat anti-SOX2 (1:500, Santa Cruz #sc-17320), rabbit anti-S100 (1:500, Abcam #ab868). Alex Fluor (488, 546, and 633) labeled secondary antibodies were used to visualize staining (1:1000, Molecular Probes/ Life Technologies). Stereocilia were visualized with fluorescently labeled phalloidin (1:500, Molecular Probes/ Life Technologies).

Immuno-staining protocol:

1. Wash slides/whole tissues 3x in PBS (5 minutes each).
2. Block and permeabilize
    a. If whole mount 0.5% Triton-X with 10% donkey serum 30 min-1 hour at RT
    b. If sections 0.5% Triton-X with 10% donkey serum 30 min-1 hour at RT
3. Dilute antibody (the recommended dilution) in PBS. Incubate overnight at 4°C (For sections-500ul per section and in humidified chamber).
4. Wash slides 3x in PBS (10 minutes each).
5. Incubate in secondary antibody (in PBS) for 1 hr at RT.
6. Wash 4x in PBS (10 minutes each).
7. Mount

Quantification of hair cells and supporting cells:

Cell counts were performed in cochlear whole mounts (surface preparations) immuno-stained for hair cell marker MYO7A and supporting cell marker SOX2. Hair cell and supporting cell subtypes were identified by their location within the sensory epithelium. Two low power confocal z-stacks through the hair cell layer and the corresponding supporting cell layer were taken in the cochlear base, mid and apex. The length of the imaged segment was analyzed using Image J (http://imagej.nih.gov/ij) and the number of hair cells and supporting cells was manually counted in Photoshop CS6. A minimum of three cochleae obtained from different animals was analyzed for each genotype. To test for significance we used the paired student T-Test to obtain P-values. P values less than or equal to 0.05 were considered significant.

Figure 3.1: Newly identified Notch-regulated genes are significantly reduced in DnMAML1 Notch hypomorphic cochlea. (A) Schematic of experimental approach using forced DnMAML1 expression to disrupt canonical Notch signaling in the differentiating cochlea. (B-G) Auditory hair cell and supporting cell phenotype of Pax2-Cre; Rosa26$^{DnMAML1/+}$(DnMAML1) mutants and wild type (control) littermates stage E18.5. Abbreviations: inner hair cells (IHC), outer hair cells (OHC), border cell (BC), phalangeal cell (PC), inner pillar cells (IPC), outer pillar cells (OPC), and Deiters cells (DC), greater epithelial cells (GER). Scale bars for all panels, 20μm. (B-E) Maximum z-projections of hair cell (B, C) and corresponding supporting cell layer (D, E) in control (B, D) and DnMAML1(C, E) expressing cochlear surface preparations. Anti-myosinVIIa (MYO7A, blue) labels hair cells; phalloidin (red) labels actin-rich hair cell bundles, anti-Sox2 staining (SOX2, green) labels supporting cell and GER cell nuclei. (F-G) Cross-section of control (F) and DnMAML1 (G) cochleae; MYO7A (red) labels hair cells, SOX2 (green) labels supporting cells and GER cells. (H-I) Quantification of inner (IHC) and outer hair cell (OHC) (H) as well as supporting cell (IPC,OPC,D1,D2,D3) density in the basal region of the wild-type and DnMAML1 mutant cochlea stage E18.5. Data expressed as mean ± SEM (n = 3, *$p \leq 0.05$ was considered significant). (J) Notch target gene expression in DnMAML1 mutant (red bar) and wild-type cochlear epithelia (grey bar) stage E18.5. Relative transcript levels were analyzed using RT-qPCR. Data expressed as mean ± SEM (n = 3 biological replicates; p-value > 0.05 was considered not significant (n.s.)).

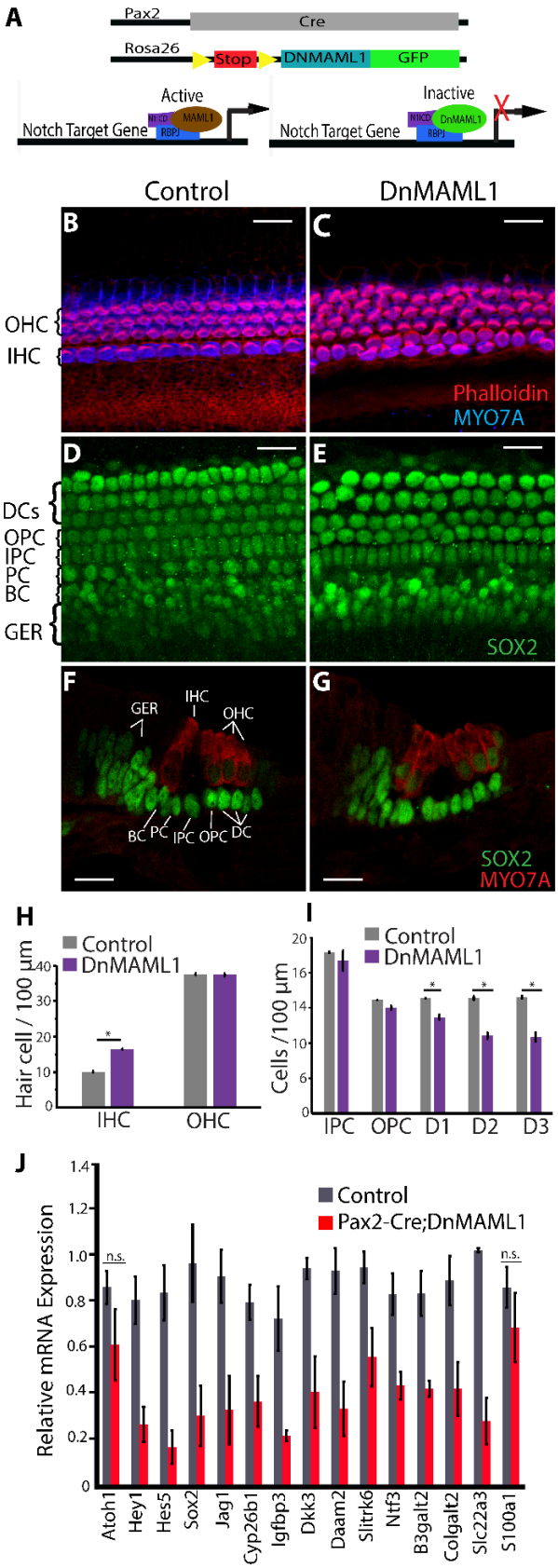
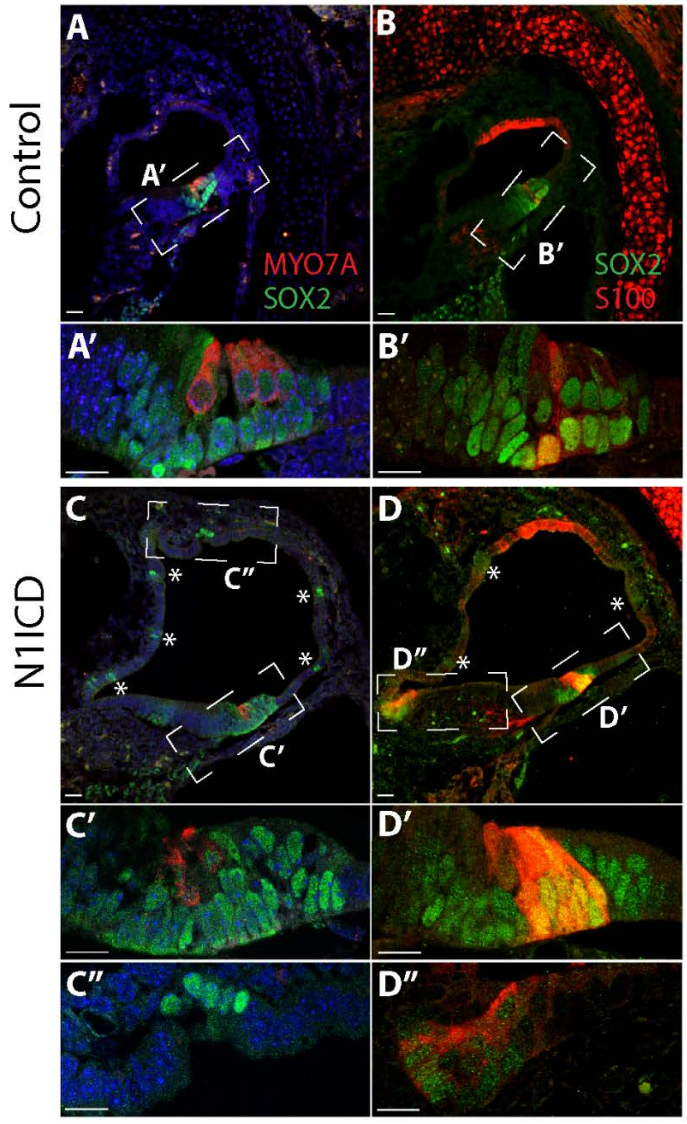

A
Pax2
Cre
Rosa26
Stop
DNMAML1
GFP
Active
Notch Target Gene
MAML1
RBPJ
Inactive
NICD
DnMAML1
Control
DnMAML1
B
C
OHC
IHC
Phalloidin
MYO7A
D
E
DCs
OPC
IPC
PC
BC
GER
SOX2
F
G
GER
IHC
OHC
BC
PC
IPC
OPC
DC
SOX2
MYO7A
H
Control
DnMAML1
Hair cell / 100 µm
IHC
OHC
I
Control
DnMAML1
Cells /100 µm
IPC
OPC
D1
D2
D3
J
Control
Pax2-Cre;DnMAML1
Relative mRNA Expression
n.s.
n.s.
Atoh1
Hey1
Hes5
Sox2
Jag1
Cyp26b1
Igfbp3
Dkk3
Daam2
Slitrk6
Ntf3
B3galt2
Colgalt2
Slc22a3
S100a1

Figure 3.2: Ectopic Notch activation promotes supporting cell-specific gene expression. (A-D") Hair cell (A, C) and supporting cell phenotype (B, D) of Emx2 $^{Cre/+}$; Rosa26 $^{N1ICD/+}$ mutants (N1ICD; C, D) and their wild type (control; A, B) littermates stage E18.0 was analyzed in adjacent cochlear sections. Hair cells are marked by anti-myosinVIIa (MYO7A red) staining; anti-Sox2 staining (green) marks supporting cells and GER cells within the sensory domain. Deiters' cells and pillar cells are marked by anti-Sox2 (green) and anti-S100 (red) staining. Hoechst staining (blue) labels cell nuclei. Dashed white lines indicate location of corresponding high power confocal images. No ectopic hair cells are observed in N1ICD over-expressing cochlea (C"); however the number of outer hair cells is reduced (C, C') compared to control (A, A') and ectopic $S100^{+}$ $SOX2^{+}$ cells are observed in the outer hair cell domain (D') and non-sensory epithelium (D") but not in control (B, B'). (e) RT-qPCR analysis of supporting cell-specific gene expression in cochlear epithelia obtained from E18.0 Emx2 $^{Cre/+}$; Rosa26 $^{N1ICD/+}$ mutants (N1ICD, purple bars) and wild-type littermates (control, grey bars). Data expressed as mean ± SEM (n=2, biological replicates, p-value > 0.05 was considered not significant (n.s.)). Similar results were obtained in a second independent experiment.


Control
N1ICD
A
A′
B
B′
C
C″
C′
D
D″
D′
MYO7A
SOX2
SOX2
S100
E
Relative mRNA Expression
Control
N1ICD
n.s.
Igfbp3
Slc22a3
Ntf3
Slc6a14
Cyp26b1
Slitrk6
Daam2
Fabp7
Jag1
Shc3
Dkk3
HeyL
Gpr126
Inhba
S100a1
Slc1a3
Plp1
Otog
Fgfr3

CHAPTER 4

Canonical Notch signaling has multiple roles in supporting cell development

INTRODUCTION

We have shown that Notch signaling is necessary and sufficient to drive supporting cell gene expression with both *in vitro* and *in vivo* experiments. Our data suggest that Notch is important for regulating a subset of the supporting cell transcriptome. Because of this important regulatory role in supporting cells, we wanted to examine if loss of Notch signaling results in abnormalities in supporting cells.

Several previous studies have used various models to disrupt Notch signaling during inner ear development, but most of these studies focused on either pro-sensory specification of progenitors (27, 81) or the role for Notch in the lateral inhibition of the hair cell fate (83). Other than conversion to the hair cell fate, the effect of Notch signaling inhibition on the supporting cell phenotype has not been extensively studied. Previous studies looked at *Notch1* mutants or at mutations of the hair cell-specific ligands *Jag2* and *Dll1*; these studies were pivotal in describing the lateral inhibition role for Notch signaling (102). These models showed ectopic hair cell formation at the expense of supporting cells and suggest that the hair cell ligands signal through the Notch1 receptor to repress the hair cell fate. The limitation of this model is that all canonical Notch signaling is not impacted. *Notch2* and *Notch3* are two additional Notch receptors expressed in addition to *Notch1* in the developing sensory epithelium (103). The Notch ligand DNER is also expressed by hair cells (104). Supporting express the Notch ligand *Jag1*, which mediates notch signaling between supporting cells (105). The function of the other ligand and receptors have not been examined in detail. Furthermore an approach to inhibit all canonical Notch signaling during supporting cell differentiation *in vivo* has not

been performed to determine if Notch has other functions independent of the hair cell fate repression. We decided to investigate the role of canonical Notch signaling in developing supporting cells with multiple loss-of-function models. We used the DnMAML1 model to look at the effect of weakening all canonical Notch Signaling in supporting cells during development and maturation. Our previous results suggested that this model is a Notch hypomorphic mutant, indicating a weakening of canonical Notch signaling. We reasoned that this approach would be useful to look at the supporting cell phenotype in developing and maturing supporting cell because it does not have a particular bias based on deleting one of multiple receptors or ligands. We also used an *Rbpj* conditional mutant to examine the effect of shutting down all canonical Notch signaling in developing supporting cells. RBP-J is the core transcription factor that binds to DNA in the Notch transcriptional complex. This model enabled us to examine the effect of completely disrupting Notch signaling in developing supporting cells.

RESULTS:

Reduction in canonical Notch signaling results in progressive Deiters' cell loss.

To determine whether physiological levels of Notch signaling are required for the proper supporting cell differentiation and maturation, we re-examined the supporting cell phenotype in the DnMAML1 over-expressing cochlea. Our initial analysis of Pax2-Cre; Rosa26 $^{DnMAML1/+}$ mutant cochlear tissue revealed mild defects in supporting cell patterning and morphology and a mild reduction in the number of basally located outer supporting cells, namely Deiters' cells. One possibility for the loss of Deiters' cells is supporting cell-to-hair cell conversion; however, Deiters' cell loss in the DnMAML1 expressing cochleae was not accompanied by an increase in outer hair cells (Fig. 3.1 H, I). To determine whether this loss in Deiters' cells becomes more pronounced at later stages, we modified our experimental approach. We examined Emx2 $^{Cre/+}$; Rosa26 $^{DnMAML1/+}$ animals, which in contrast to Pax2-Cre; Rosa26 $^{DnMAML1/+}$ animals, survive past birth, allowing us to characterize supporting cells as they undergo postnatal differentiation and maturation. As observed in the Pax2-Cre; Rosa26 $^{DnMAML1/+}$ late embryonic cochleae, hair cells were miss-patterned in the Emx2$^{Cre/+}$; Rosa26 $^{DnMAML1/+}$ postnatal cochleae (P0 and P5), and the number of inner hair cells but not outer hair cells was significantly increased compared to wild type (control) littermates (Fig. 4.1 A-L, M, N). In contrast to the relative stable hair cell phenotype, the supporting cell phenotype significantly worsens between P0 and P5 in DnMAML1 expressing cochlear tissue (Fig. 4.1 A'-L'). At P0, Deiters' cell loss was only evident in the base of the DnMAML1 mutant cochlea (Fig. 4.1 B'). However, five days later at P5, Deiters' cells were missing

throughout the entire length of the cochlear duct in DnMAML1 mutant cochleae and the number of 2nd and 3rd row Deiters' cells was significantly reduced compared to P5 control cochleae (Fig. 4.1 O). DnMAML1 mutant Deiters' cells had a highly abnormal morphology; their cell nuclei were enlarged and their nuclear arrangement was disorganized (Fig. 4.1 H', J'), a large contrast to the stereotypical arrangement of wild-type Deiters' cell nuclei (Fig. 4.2 G', I').

To see if any further cell death occurs we examined adult Emx2-Cre DnMAML1 mutant mice. The DnMAML1 mice were smaller in appearance than the control littermate (Fig. 4.2 C) suggesting defects in either ability to gain adequate nutrition or growth. The mutant mice also exhibited bald spots due to excessive grooming (Fig. 4.2 C). We next examined the cytoarchitecture of the mature DnMAML1 transgenic cochlea. To do this we isolated cochlea from P26 stage mice, fixed the tissue and sectioned the inner ears in paraffin. We then stained the paraffin sections with hematoxylin and eosin to view the architecture an organization of the organ of Corti. The DnMAML1 cochlea had a number of defects (Fig 4.2 D, E, F, G). Some sections contained fairly normal hair cell arrangements but were missing the third Deiters' cell. The remaining Deiters' cells nuclei were misplaced and closer to the basement membrane than what is normally seen in wild type cochleae of this stage. In other sections Deiters' cells were disorganized or absent and the tunnel of Corti was collapsed. Outer hair cells were displaced or in some cases missing due to missing Deiters' cells, on which they usually sit (Fig 4.2 E, F, G). These results suggest that from the P5 stage degeneration of the sensory cells continues in the DnMAML1. Overall our data suggests that prolonged reduction in canonical Notch signaling results in progressive loss of Deiters cells.

Ablation of canonical Notch signaling causes Deiters cells to die.

To independently confirm the requirement of canonical Notch signaling for Deiters' cell development and/or survival, we decided to completely abolish Notch signaling in differentiating Deiters' cells. To do so, we utilized a conditional *Rbpj* mutant line (106). The transcription factor RBP-J is critical for the transcriptional output of all Notch receptor signaling and ablation of the *Rbpj* gene results in a complete abolishment of canonical Notch signaling within that cell. *Rbpj* has an early role in pro-sensory cell development(27, 81). To bypass the early requirement for *Rbpj* we selectively deleted *Rbpj* at later stages in Deiters' cells, pillar cells and outer hair cells using the recently developed tamoxifen inducible Fgfr3-iCreER transgenic line(107) (Fig. 4.3 A). Tamoxifen was administrated at E14.5 and E15.5 to pregnant dams, and the hair cell and supporting cell phenotype was analyzed three days later at stage E18.5 in control (Fgfr3-iCreER; Rbpj $^{\Delta/+}$) (Fig. 4.3 A, C, E-J) and *Rbpj* mutant (Fgfr3-iCreER; Rbpj $^{\Delta/-}$) littermates (Fig. 4.3 B, D, K-P). SOX2 staining revealed large gaps/holes in the *Rbpj* mutant supporting cell layer, which corresponded to missing Deiters' cells (Fig. 4.3 B, D, N, O). This decrease in Deiters' cell density in *Rbpj* mutant cochlea compared to control was not accompanied by an increase in outer or inner hair cells density, indicating that the loss of Deiters' cells was not caused by the conversion of Deiters' cells into hair cell-like cells (Fig. 4.3 Q-R). However, due to the severe loss of surrounding Deiters' cells, outer hair cells clumped together, and outer hair cell arrangement was severely disorganized in the base and mid segment of the *Rbpj* mutant cochlea (Fig. 4.3 K, L). Moreover, TUNEL staining revealed apoptotic cells within the outer supporting cell layer in *Rbpj* mutant, but not in control cochlear tissue, suggesting that in the absence of *Rbpj,*

Deiters' cell survival is compromised (Fig. 4.3 C, D). The remaining *Rbpj* mutant Deiters' cells had severely enlarged cell nuclei, indicating cellular stress and/or injury (Fig. 4.3 D, N, O). Interestingly, as earlier observed in DnMAML1 mutants, pillar cells, particularly inner pillar cells, were largely unaffected by the loss of *Rbpj* (Fig. 4.3 B, S). FGFR3 signaling, which is highly activated in differentiating pillar cells plays a key role in their differentiation (108), and it is likely that FGFR3 signaling largely compensated for the loss of Notch signaling in pillar cells. In summary, our data suggests that Deiters' cells require canonical Notch signaling for their proper development and that the abolishment of canonical Notch signaling in differentiating Deiters' cells results in their death.

Reduction in canonical Notch signaling results in innervation defects.

Supporting cells have been shown to be important for spiral ganglion development (8, 31, 75, 109). Because supporting cells are abnormal in the DnMAML1 model we wanted to see if reduction of Notch signaling causes defects in neuronal innervation. Additionally the list of Notch regulated genes included genes with functions in neuronal guidance and innervation (*Ntf3* and *Slitrk6),* further warranting an examination of the innervation in Notch mutants To do this we, we stained DnMAML1 mutant and control cochlear tissue stage P0 and P5 for neurofilament using an anit-neurofilament H antibody. This antibody allows us to label the neuronal fibers that innervate the sensory cells. In the P5 DnMAML1 mutant we observed that the fibers that innervate the outer hair cells were heavily disorganized (Fig4.6 D', H' L'). At this stage

the supporting cells have already begun to degenerate so one possibility for the disruption is the loss of the supporting cells and abnormal cyto-architecture. We examined the P0 cochlea to see if defects in outer hair cell innervation occur prior to the loss of the supporting cells. At P0 outer supporting cells are largely intact; we still see a disruption in the innervation pattern (Fig 4.5 B' F, J'). Since the innervation phenotype precedes the majority of the Deiters' cell death we can conclude that disruption of Notch and not death of supporting cells is causing the innervation phenotype. This is an interesting phenotype and additional experiments will be needed to determine the exact mechanism causing the observed innervation defects.

DISCUSSION

Previous work has shown that Notch signaling is primarily important for lateral induction of pro-sensory progenitors and the repression of the hair cell fate in the developing supporting cells. Here we present new work that suggests additional functions for canonical Notch signaling in supporting cell development. Based on our mutant models we conclude that Notch signaling is important for restricting the hair cell fate, is critical for the survival of developing supporting cells, positively regulates a supporting cell specific gene expression program and is important for proper innervation of the cochlea.

We found that an additional role for Notch signaling is to promote the survival of developing supporting cells. All canonical Notch Signaling uses the RBP-J and MAML proteins to activate expression of target genes. RBP-J and MAML proteins are critical components of the Notch trans-activation complex. Genetic *Rbpj* ablation or forced expression of the dominant negative MAML1 (DnMAML1) protein disrupts or reduces transcriptional activation by all canonical Notch signaling. Forced expression of DnMAML1 at the onset of cochlear differentiation as well as conditional deletion of *Rbpj* in the differentiating cochlea resulted in a significant loss of Deiters' cells. In these Notch mutant mouse models the reduction in the number of Deiters' cells was not due to supporting cell-to-hair cell conversion as no corresponding increase in the number of outer cells was observed. This is in stark contrast to *Notch1* receptor mutants, in which outer hair cells are overproduced at the cost of Deiters' cells (102). Instead, the presence of TUNEL positive Deiters' cells in E18.5 *Rbpj* mutants suggests that in the absence of

all canonical Notch signaling, differentiating Deiters' cells initiate an apoptotic or necrotic-like process and die. This result leads one to ask why Deiters' cells are dying when Notch signaling is disrupted. One plausible explanation is that in the absence of Notch signaling genes critical for supporting cell differentiation are deregulated or lost, causing supporting cells to die. Alternatively, Notch signaling might be required for the expression of a pro-survival gene(s), which once lost results in cell death. A third mechanism could result from the up-regulation of pro-apoptotic genes due to the loss of the repressive function of RBP-J. The finding that Notch signaling is critical for cell survival is observed in other cellular contexts. One example is in chronic lymphocytic leukemia (CLL), where Notch signaling sustains survival by activating *Mcl-1* (110).

In *Rbpj* as well as DnMAML1 mutants, Deiters' cells and outer pillar cells located in the base were more affected than Deiters' cells and outer pillar cells located further apically. Moreover, Deiters' cells located at the lateral edge of the sensory epithelium were more affected than the more medially located outer pillar cells. One explanation for this differential response could be that cells at the lateral edge require higher levels of Notch signaling and are more sensitive to its disruption. An analysis of Notch signaling levels in different supporting cell subtypes has not yet been and would be necessary to determine if there are differential levels on Notch activity in different supporting cell subtypes. An alternative explanation for this graded response is the existence of additional signals that modulates Notch dependency in differentiating Deiters' cells and outer pillar cells. Potential candidates are FGFR3 signaling and Shh signaling, both of which have been recently reported to modulate Notch dependency and Notch dependent gene expression in pillar cells and apical pro-sensory cells respectively (29, 53). Finally,

the phenotypic differences between *Notch1* mutant, *Jag1* mutant, and *Rbpj* (and DnMAML1) mutant cochleae, suggests that the instructive function of Notch signaling in supporting cell differentiation is mediated by a distinctly different set of Notch signaling components than its repressive function, or that specific combination of Notch components are required for each function.

Our observation that reduced Notch signaling results in abnormal innervation of outer hair cells suggests that Notch signaling is important for the proper development of innervation. Since the defects in innervation precede the loss of the majority of the outer supporting cells we do not believe that this phenotype is related to the cell death. This is an interesting observation but we do not know how exactly Notch signaling is involved in development of innervation. We uncovered two Notch regulated genes (*Slitrk6* and *Ntf3*) that are important for development of innervation. One plausible mechanism would be that the loss of expression of these genes in the Notch mutant is responsible for the disruption. More experiments will be needed to determine exactly how Notch is involved in this process.

Overall we have shown that in addition to its classic role in hair cell fate repression, Notch signaling play an instructive role in supporting cell development by regulating the supporting cell-specific gene program. These positively regulated genes have diverse functions such as transcription factors or modulators of powerful cell signaling pathways. By regulating these genes, Notch signaling could be influencing the differentiation of the supporting cells. Other genes regulated by Notch include channel and transport proteins that could be import for the supporting cell's ability to modulate ion and small molecule homeostasis, suggesting that Notch signaling is directly

regulating genes important for supporting cell function. We also find that loss or reduction of canonical Notch signaling results in defects in outer supporting cell viability. This suggests that Notch signaling is critical for supporting cell survival. We also observe a disruption in innervation when Notch signaling is disrupted indicating it may play a role in this process. Taken together our findings indicate that Notch signaling has important functions in supporting cell development beyond repressing the hair cell fate. Additional experiments are needed to determine which Notch components are involved the different roles Notch plays in inner ear development.

MATERIALS AND METHODS

Mouse breeding and genotyping:

All experiments and procedures were approved by the Johns Hopkins University Institutional Animal Care and Use Committees protocol, and all experiments and procedures adhered to National Institutes of Health-approved standards. The Fgfr3-iCreER PAC transgenic line was obtained from William Richardson (UCL, London, UK)(111). Conditional Rbpj $^{fx/fx}$ and conventional Rbpj$^{-/+}$ knock out lines were obtained from Tasuku Honjo (University of Kyoto, Kyoto, Japan) (106). Mice were genotyped by PCR as previously described for each line. Mice of both sexes were used in this study. All mouse lines were maintained on a mixed background of C57BL/6 and CD-1. To conditionally delete Rbpj, Rbpj $^{fx/fx}$ mice were timed mated with the Fgfr3-iCreER/Ai14; Rbpj$^{-/+}$ mice and the pregnant dams received a single injection of tamoxifen (0.125mg/g body weight, Sigma) and progesterone (0.125mg/g body weight, Sigma) at E14.5 and E15.5 and Rbpj mutant (Fgfr3-iCreER/Ai14; Rbpj $^{\Delta/-}$) embryos and their wild type littermates (Fgfr3-iCreER/Ai14; Rbpj $^{\Delta/+}$) were analyzed at E18.5.

In situ cell death assay (TUNEL staining)

To detect dying cells, an in situ cell death detection kit- fluorescein was used according to manufactures instructions (Roche Diagnostics, GmbH).

Tissue processing for paraffin sectioning

Cochleae were dissected from the mutant and control littermates. Tissue was fixed with a 4% paraformaldehyde 2% glutaraldehyde solution in 1x PBS by injection the solution through the round window membrane. After injecting the fixation solution into the cochlea the entire cochlea was submersed in fixative and incubated at 4^0 C overnight. The next day samples were washed with 1x PBS and then decalcified with 14% EDTA in 1xPBS for three days. Once decalcified, samples were embedded in paraffin cochleae were sectioned at 10 μm. To visualize the cellular structure slides were stained with hematoxylin and eosin. The stained slides were analyzed with light microscopy.

Figure 4.1: Prolonged reduction of canonical Notch signaling by DnMAML1 expression results in a progressive loss of outer supporting cells.

Hair cell and supporting cell phenotype in Emx2 $^{Cre/+}$ Rosa26 $^{DnMAML1/+}$ (DnMAML1) mutants and their wild type (control) littermates, stage P0 and P5. (A-L') Maximum z-projections of control (P0: A, C, E; P5: G, I, K) and DnMAML1 (P0: B, D, F; P5 H, J, L) mutant hair cell layer (MYO7a, red) and corresponding supporting cell layer (') (SOX2, green). Shown are basal (A-B', G-H'), mid (C-D', I-J') and apical (E-F', K-L') fields. (M-O) Quantification of inner hair cell (M), outer hair cell (N) and outer supporting cell (O) density in the cochlear base, mid and apex of control (P0, light grey; P5 dark grey) and DnMAML1 mutant (P0 purple; P5 blue) animals. Abbreviations: inner pillar cells (IPC), outer pillar cells (OPC), and Deiters cells row 1, 2 and 3 (DC1-3). Data expressed as mean ± SEM (n=3, biological replicates, *$p \leq 0.05$ was considered significant). Scale bars for all panels, 20μm.

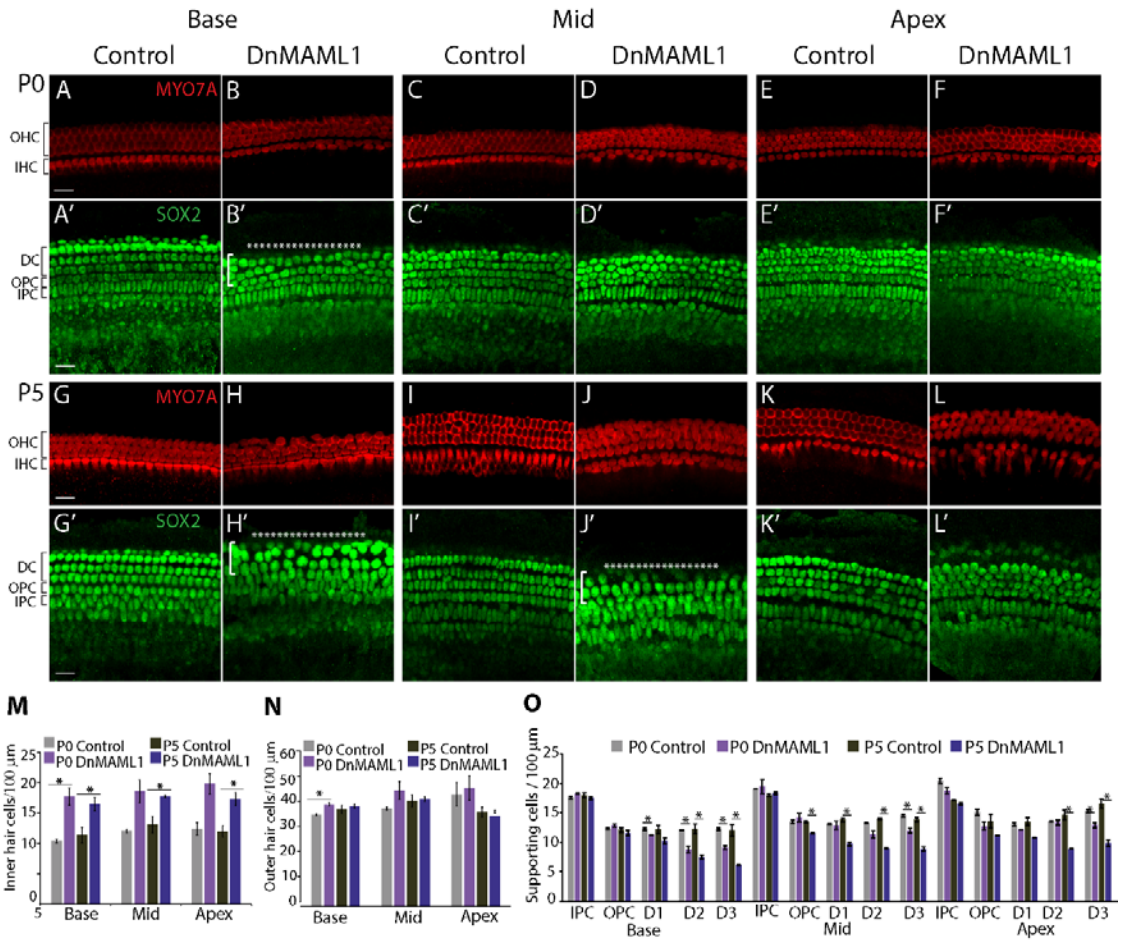

Base
Mid
Apex
Control
DnMAML1
P0
P5
MYO7A
SOX2
OHC
IHC
DC
OPC
IPC
M
N
O
P0 Control
P0 DnMAML1
P5 Control
P5 DnMAML1
Inner hair cells/100 µm
Outer hair cells/100 µm
Supporting cells / 100 µm
IPC
OPC
D1
D2
D3
Base
Mid
Apex

Figure 4.2: The loss of outer supporting cells worsens as DnMAML1 mice reach maturity

(A, B) Hematoxylin and eosin stained sections of control adult cochlea showing the proper arrangement of inner and outer hair cells and supporting cells at postnatal day 26. Hematoxylin stains nuclei blue and eosin stains other cellular structures various shades of pink or red. (C) Photograph of control ($Emx2^{+/+;}$ $Rosa26^{DnMAML1/+}$) (left) and DnMAML1 mutant ($Emx2^{Cre/+;}$ $Rosa26^{DnMAML1/+}$) (Right) mice at postnatal day 26. The DnMAML1 mouse is significantly smaller that its control littermate. (D- G) Hematoxylin and eosin stained sections of DnMAML1 cochlea ($Emx2^{Cre/+;}$ $Rosa26^{DnMAML1/+}$). The sections reveal various degrees of outer hair cell and supporting cells loss. Sections E, F and G show a collapse in the tunnel of corti. Deiters' cells are missing in sections E, F and G. In section D the remaining Deiters' cells are misplaced.

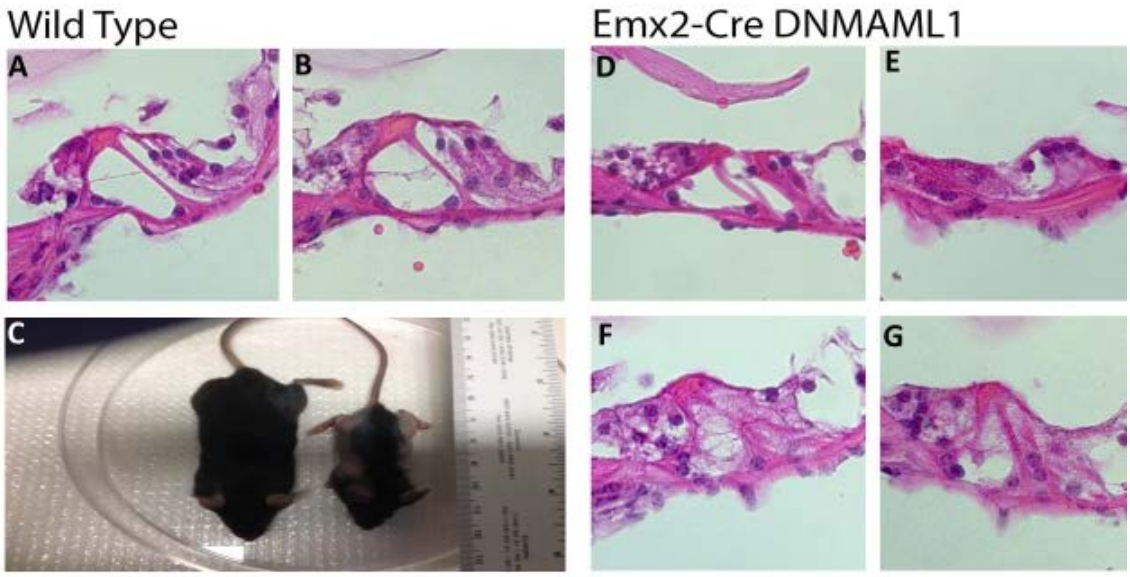

Wild Type
Emx2-Cre DNMAML1
A
B
C
D
E
F
G

Figure 4.3: Ablation of canonical Notch signaling by deleting Rbpj results in a loss of outer supporting cells.

Tamoxifen was injected at E14.5 and E15.5 and cochlear hair cell (MYO7A) and supporting cell phenotype (SOX2) in Fgfr3-iCreER; Ai14; Rbpj $^{\Delta/+}$ (control) and of Fgfr3-iCreER; Ai14; Rbpj $^{\Delta/-}$ (Rbpj mutants) littermates was analyzed at stage E18.5. (A, B) Shown are high-power confocal images of mid-basal cochlear sections from control (A) and Rbpj mutant (B) animals. Ai14 Cre-reporter (Td-tomato, purple) reveals cells in which Cre-mediated recombination occurred. Note that Deiters cells (D1-D3) but not outer pillar cells (OPC) or inner pillar cells (IPC) are missing in Rbpj mutants (B). (C, D) High-power confocal images of supporting cell layer in control (C) and Rbpj mutant cochlear surface preparations (B). SOX2 marks supporting cell nuclei (red) TUNEL staining reveals apoptotic/ necrotic cell nuclei (green). Asterisks mark TUNEL+ Deiters cells in the Rbpj mutant (D) cochlear sensory epithelia. (E-P) Maximum z projections of hair cell layer (MYO7A, red) and supporting cell layer (SOX2, green) of control (E-J) and Rbpj mutant (K-P) cochlear surface preparations at the indicated position. (O-Q) Quantification of inner hair cell (O), outer hair cell (P) and outer supporting cell (IPC, OPC, D1-3) (Q) density in the cochlear base, mid and apex of control and Rbpj mutant animals. Data expressed as mean ± SEM (n=3, biological replicates, *$p \leq 0.05$ was considered significant). Scale bars for all panels, 20μm

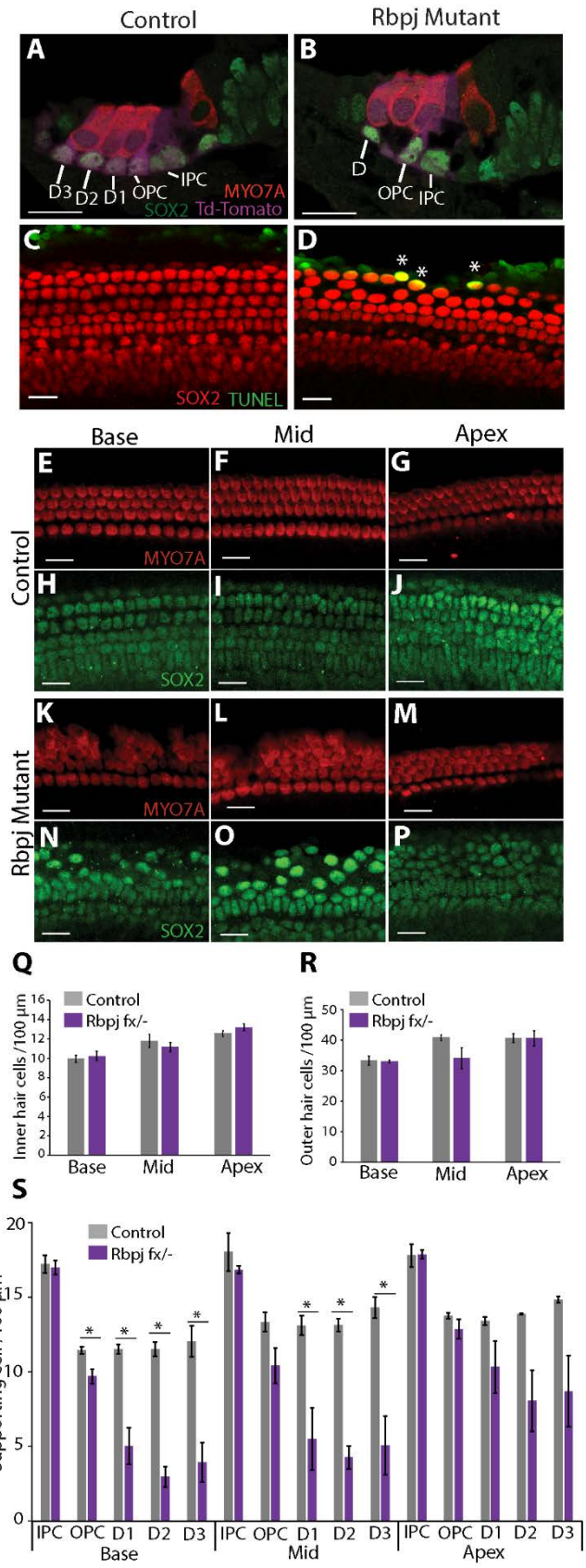
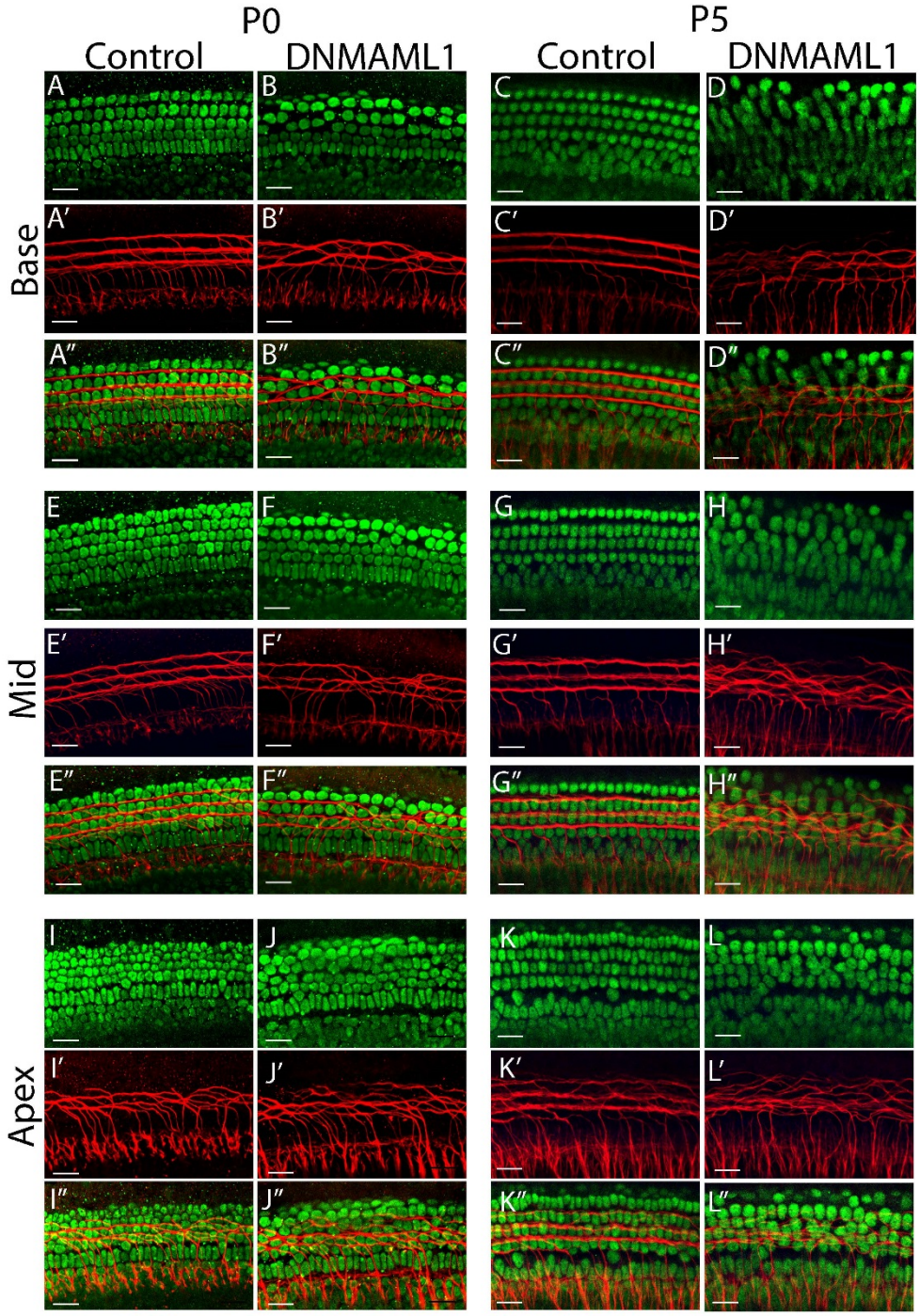

Control
Rbpj Mutant
A
B
C
D
D3
D2
D1
OPC
IPC
MYO7A
SOX2 Td-Tomato
D
OPC
IPC
SOX2 TUNEL
Base
Mid
Apex
E
F
G
H
I
J
K
L
M
N
O
P
Control
Rbpj Mutant
MYO7A
SOX2
Q
R
S
Control
Rbpj fx/-
Inner hair cells /100 µm
Outer hair cells /100 µm
Supporting cell /100 µm
Base
Mid
Apex
IPC OPC D1 D2 D3

Figure 4.4: Innervation defects in the Emx2-Cre DnMAML1 cochlea.

Confocal images of representative fields of the basal, mid and apical segment of DnMAML1 mutant ($Emx2^{Cre/+}$; $Rosa26^{DnMAML1/+}$) and wild type sensory epithelium ($Emx2^{+/+}$; $Rosa26^{DnMAML1/+}$) stage P0-P5. (A-J) Anti-SOX2 staining (green) marks supporting cell nuclei. Note the gradual degeneration of supporting cells in the DnMAML1 mutant cochlea between P0 and P5. (A'-J') Anti-neurofilament staining (red) marks neuronal fibers. (A"-J") Merge showing both neurofilament (red) and SOX2 staining (green). Note the disorganization of the neuronal innervation in the DnMAML1 mutant auditory sensory epithelium. In the wild type cochlea, neuronal fibers turn uniformly towards the base and run in three parallel tracks underneath the outer hair cells. In the DnMAML1 mutant cochlea fibers turn in both directions and cross over each other at a greater frequency Scale bars 20μm in all panels.


P0
P5
Control
DNMAML1
Control
DNMAML1
Base
Mid
Apex
A
B
C
D
A′
B′
C′
D′
A″
B″
C″
D″
E
F
G
H
E′
F′
G′
H′
E″
F″
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I
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CHAPTER 5

Different Notch signaling components mediate the multiple functions of Notch signaling in the developing cochlea

INTRODUCTION

Previous work revealed distinct functions for Notch ligand, *Jag1,* and receptor, *Notch1,* in pro-sensory development and hair cell fate repression. These different phenotypes suggest that Notch signaling generated by different Notch ligand and Notch receptor combinations might evoke different cellular responses. In this chapter we explore the functions of the Notch receptor *Notch1* and the Notch ligand *Jag1* in the differentiating cochlea to see how they contribute to the roles that canonical Notch signaling plays in developing supporting cells. Previous studies showed that in the *Jag1* mutant (Foxg1-Cre Jag1fx/fx) cochlea, inner hair cells are overproduced but outer hair cells and the surrounding Deiters' cells and are missing. This defect, even though more extreme, resembles the postnatal phenotype of the DnMAML1 mutant cochlea. However, a caveat with these experiments is that due to the early deletion of Jag1 (~E9.0) it is unclear when these defects arise, and what cellular processes are disrupted (e.g sensory specification, survival). To be able to compare the phenotypes that arise from ablation of *Jag1* and *Notch1* with our DnMAML1 mutant findings, we generated Emx2-Cre Jag1 fx/fx and Emx2-Cre Notch1fx/fx mutant lines and analyzed their supporting cell and hair cell phenotypes. Finally we examine gene expression in the *Jag1* mutant to see if Jag1 is involved in the regulation of the newly identified Notch regulated genes.

RESULTS

Notch1 is critical for hair cell fate repression but is dispensable for supporting cell survival

Emx2-cre induces recombination around E13.0 in auditory sensory progenitors (37). $Emx^{Cre/+;}$ $Notch1^{fx/fx}$ mice survive until early postnatal stages unlike previous *Notch1* mutants (50). We analyzed mutant and control littermates at postnatal day P4. In the control we observed the stereotypical pattern of one row of MYO7A+ inner hair cells and three rows of MYO7A+ outer hair cells (Fig 5.1 A, C). In cross-section we see the normal compliment of SOX2+ supporting cells in their stereotypical arrangement (Fig. 5.1 A). In the *Notch1* mutant we see an excessive over-production of both inner and outer hair cells (Fig5.1 B, D). These hair cells are disorganized and in multiple layers as opposed to one single layer. They are also atypically arranged and contact each other because there no longer are supporting cells. Most of the SOX2+ supporting cells are lost, presumable due to hair cell conversion (Fig 5.1 B). Pillar cells are the only supporting cells visible in the organ of Corti. From this experiment we conclude that *Notch1* primarily functions to repress the hair cell fate. Other Notch signaling components (specifically another receptor) or combination of components therefore must be critical for the pro-survival function, because both the inner and outer hair cell regions form in *Notch1*-deficient mice.

Jagged1 is involved in repressing the hair cell fate in inner supporting cells and survival of outer hair cells and outer supporting cells

Next we used the Emx2-Cre line to delete *Jag1* just prior to hair cell and supporting cell differentiation. The *Jag1* mutant resulted in a phenotype that was dramatically different from the *Notch1* deletion at the same stage. In the $Emx2^{cre/+}$; $Jag1^{fx/fx}$ we observed ectopic inner hair cells and a loss of the majority of the outer hair cells (Fig 5.2 B'). The ectopic inner hair cells indicate that *Jag1* is involved in repressing the hair cell fate in the inner supporting cell region. Interestingly, outer hair cells and their surrounding Deiters' cells were missing as shown by the loss of SOX^{+} and $S100^{+}$ cells on the lateral area of the auditory sensory epithelium (5.2 B" B'''). This result suggests that *Jag1* is critical for the survival of the lateral sensory progenitors or the survival of their progenies, the differentiating outer hair cell and supporting cells. Since the *Notch1* mutant does not reveal a loss of the lateral region of the auditory sensory epithelium, we can conclude that an alternative Notch receptor is mediating this process through its interaction with *Jag1*.

Jagged 1 mediated Notch signaling is required for the proper expression of a subset of Notch regulated genes during supporting cell differentiation.

We have shown that *Jag1* is critical to the survival of cells of the lateral sensory domain. *Jag1* continues to be expressed in supporting cells and is thought to mediate Notch signaling between supporting cells (105). To determine potential later roles of *Jag1* in supporting cell differentiation we used Sox2-CreER, a supporting cell-specific tamoxifen inducible cre line to ablate *Jag1* in differentiating supporting cells. We

administered tamoxifen at E14 and E15 to pregnant dams and harvested *Jag1* mutant (Sox2-CreER; Jag1fx/fx) and control (Jag1 fx/fx) littermates at E18.5. Inducing recombination at this stage should bypass the earlier requirement for *Jag1* in pro-sensory cell survival. The control littermates had a normal compliment of hair cells and supporting cells in the apical, mid, and basal regions (Fig5.3 A- C'). The $Sox2^{Cre/+}$; $Jag1^{fx/fx}$ mice had a few ectopic outer hair cells in the basal region and ectopic inner hair cells in the base, mid and apical regions (Fig5.3 D- F) confirming its role in repressing the hair cell fate. The supporting cell arrangement in Jag1 mutant cochlea was mildly disorganized compared to control littermate (Fig5.3 D' E' F'). We observe the most disorganization in the apical region (Fig5.3 D'). We did not observe a defect in supporting cell survival in E18.5 Sox2-CreER Jag1 fx/fx mutant cochlea, suggesting that at later stages hair cell-specific Notch ligands compensate for the loss of *Jag1* and maintain supporting cell survival in the absence of *Jag1*.

Next we wanted to see if *Jag1* is important for the regulation of the Notch regulated genes we identified in the microarray experiment and validated with the DnMAML1 model. To look at the genes regulated by Jagged1-mediated Notch signaling, we isolated RNA from control and *Jag1* mutant cochlear epithelia. We then preformed RT-qPCR to look at changes in levels of select Notch responsive genes as compared to the control. We first examined levels of *Jag1* and found it to be reduced to less than 0.15 in the *Jag1* mutant cochlea compared to control, confirming successful *Jag1* ablation (Fig 5.3 G). We found that some Notch target genes were unchanged in the mutant such as *Fgf20* and *Inhba*. We found some of the Notch target genes to have a modest decrease in their expression level (*Ntf3, Slitrk6, Nrcam, Daam2 Gucy1b3*). This suggests that *Jag1* is

partially regulating these genes but other Notch ligands are also co-regulating their expression. We then found a few genes that had a large decrease in their expression levels (*Elf5, Igfbp3*). This finding suggests that *Jag1* could be the primary ligand regulating these supporting cell specific genes. Overall, our data suggests that different ligands or combinations of ligands could be differentially regulating supporting cell-specific genes.

DISCUSSION

By contrasting the differing phenotypes of conditional *Jag1* and *Notch1* mutants we provide evidence that different Notch components contribute to different functions of canonical Notch signaling. We confirmed that *Notch1* functions mostly in repression of the hair cell fate in developing supporting cells. We showed that deletion of *Notch1* just prior to differentiation revealed the same phenotype that was previously observed (50). This finding leads us to conclude that *Notch1* and its interaction with *Dll1* and *Jag2* are critical for the repressive function of Notch signaling (102). Previous work with *Dll1* and *Jag2* mutants indicates that these ligands also function in hair cell fate repression, presumable by activating Notch1 (102). This conclusion is based on the similarities between the hair cell ligand compound mutants and the *Notch1* mutant. This result also suggests that a different Notch ligand is important for the survival of the outer sensory cells that are lost in the $Emx2^{Cre/+}$; $Jag1^{fx/fx}$ mutant. Both *Notch2* and *Notch3* are expressed in the developing cochlea and individually or cooperatively these Notch receptors may mediate the pro-survival function of Notch signaling (103).

Typically Notch signaling is thought of as a signal from hair cells to newly developing supporting cells. *Jag1* is unique among Notch ligands because it is expressed early in progenitors and later in supporting cells. Interestingly, *Jag1* is not only a Notch ligand but also a Notch target gene (105). The function of *Jag1* in supporting cells is currently unknown.

Early on *Jag1* functions in lateral induction of pro-sensory progenitors (26). During this phase of Notch signaling *Jag1* is the only Notch ligand expressed; *Jag1* mutants revealed a loss of sensory cells, thus implicating in specification of pro-sensory

progenitors (26). Subsequent studies used an *Rbpj* deletion to investigate the involvement of Notch signaling in pro-sensory specification. In these studies pro-sensory markers were induced normally but as soon as hair cells and supporting cells started to differentiate they would die leading to the interpretation that Notch signaling is necessary for maintaining, but not specifying, the pro-sensory progenitors (27). Since *Jag1* is the sole Notch ligand expressed at this time, it presumably is the Notch ligand mediating the survival of these cells. The Emx2-Cre mediated deletion of *Jag1* supports this interpretation because it has a similar phenotype to the early *Rbpj* deletion. Sensory cells are induced but because the lateral portion of the organ of Corti is missing, we reason that *Jag1* is critical for the survival of these cells.

As differentiation progresses additional Notch ligands become expressed which makes determining the role of *Jag1* at this time point more complicated. A previous study using antisense oligonucleotides implicated *Jag1* in repression of the hair cell fate (35). Since the methodology of using antisense oligonucleotides fell out of favor, the field largely ignored this study in favor of other studies that used genetic ablation of *Jag1*. The problem with these previous studies is that they disrupted *Jag1* at a very early stage, when it is the only ligand expressed, so it is impossible to observe its function when multiple ligands are mediating Notch signaling in the differentiating cochlea. Both of our *Jag1* models confirm that Jag1 is involved in hair cell fate repression, most notably in the inner supporting cell region. When we deleted *Jag1* at E14.5, we continue to see its requirement for hair cell fate repression but at this stage we no longer observe a cell death phenotype. This is in contrast to what we see when we disrupt all canonical Notch signaling by deleting *Rbpj* at this stage. Because *Dll1*, *Jag2*, *Dner*, and *Dll3* are expressed

at this stage we reason that these other ligands can compensate for the loss of *Jag1* and maintain the survival of the developing supporting cells since *Jag1* is dispensable for this function at this stage (49, 112, 113).

Interestingly when we examined changes in gene expression of Notch regulated genes in the Sox2-CreER Jag1 mutant we found some genes to be more dependent on *Jag1* than others. This suggests that in the mammalian system, different Notch ligands can potentially regulate different Notch target genes. This observation was first observed in the avian system and we now show this for the first time in the mammalian system (114). In the avian system, it has been shown that *Jag1* and *Dl1* induce different levels of Notch activity. This differential in levels of Notch activity is what accounts for the differential regulation of target genes (114). This finding suggests that the activity of Notch ligands, or signal strength is involved in the transcriptional activation of Notch target genes. Deleting *Jag1* alters Notch signaling strength and results in a change in Notch transcriptional activation. This finding suggests that there is an incredible amount of complexity in how Notch signaling differentially regulates different target genes in the developing cochlea. A further complication in the mammalian system is that instead of having a single Notch receptor, multiple Notch receptors are expressed, adding yet another layer of complexity that could result in differential regulation of target genes. Additional experiments are required to determine the precise mechanism for how this differential regulation of gene expression is achieved in the mammalian inner ear.

MATERIALS AND METHODS

Mouse breeding and genotyping:

All experiments and procedures were approved by the Johns Hopkins University Institutional Animal Care and Use Committees protocol, and all experiments and procedures adhered to National Institutes of Health-approved standards. The Notch1 conditional mutant line (006951) was purchased (Jackson Laboratory, Bar Harbor, USA). Jag1 mice were obtained from Julian Lewis (University College London, United Kingdom). Sox2-CreER mice (017593) were purchased from Jackson Laboratory Bar Harbor, USA. Mice were genotyped by PCR as previously described for each line. Mice of both sexes were used in this study. All mouse lines were maintained on a mixed background of C57BL/6 and CD-1. To conditionally delete Jag1, Jag1 $^{fx/fx}$ mice were timed mated with the Sox2$^{CreER/+}$; Jag1$^{fx/fx}$ mice and the pregnant dams received a single injection of tamoxifen (0.125mg/g body weight, Sigma) and progesterone (0.125mg/g body weight, Sigma) at E14.5 and E15.5 and Jag1 mutant embryos and their wild type littermates were analyzed at E18.5.

Figure 5.1: Notch1 is the main Notch receptor involved in lateral inhibition

We used Emx2-Cre to drive the deletion of *Notch1* just prior to the onset of hair cell and supporting cell differentiation. (A) Cross-section of control ($Emx2^{+/+}$; $Notch1^{fx/fx}$) littermate showing MYO7A+ hair cells in green and SOX2+ supporting cells in red. Normal pattering is observed. (B) Whole mount view of MYO7A+ hair cells shows one row of inner hair cells and three rows of outer hair cells in the control littermate. (D) Cross-section of Notch1 mutant ($Emx2^{Cre/+}$; $Notch1^{fx/fx}$) that shows an overproduction of MYO7A+ hair cells (Green) at the expense of SOX2+ supporting cells (Red). (E) Whole mount view of Notch1 mutant showing MYO7A+ hair cells. There is an extreme overproduction of both outer and inner hair cells. Stage analyzed is postnatal day 3 (P3). Scale bar is 20 μm in all panels.

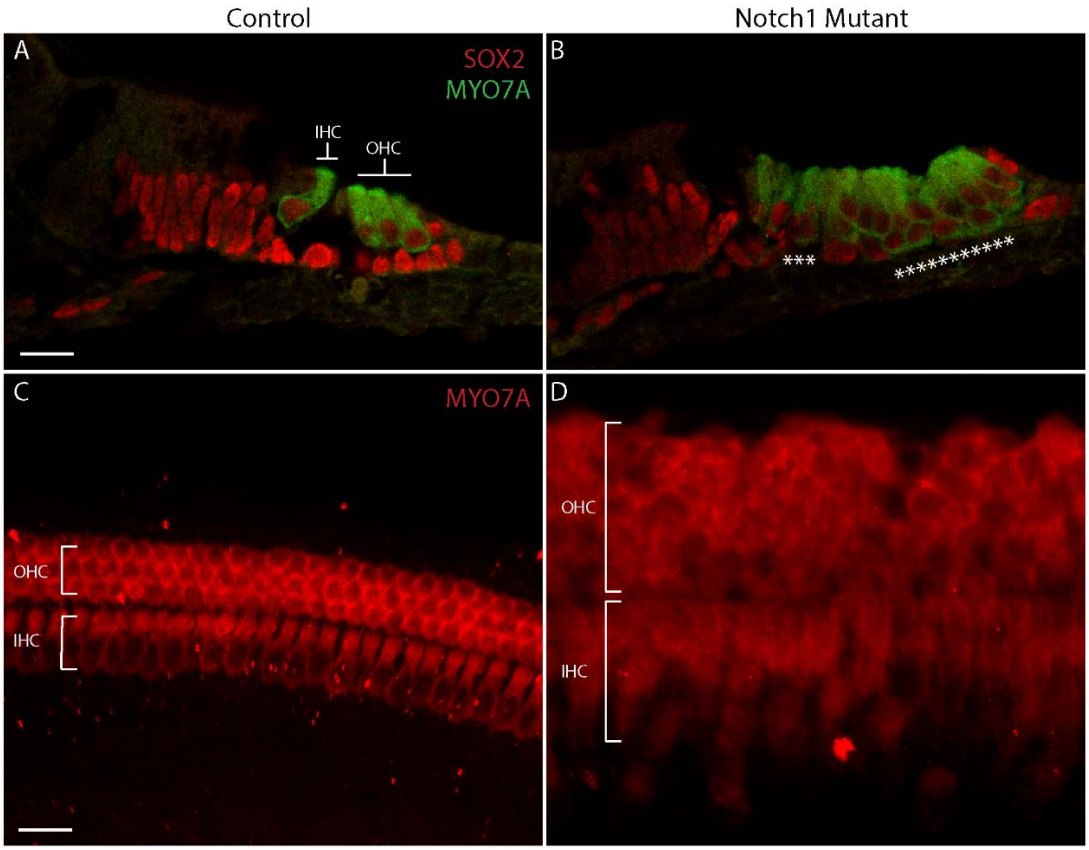

Control
Notch1 Mutant
A
SOX2
MYO7A
IHC
OHC
B
***
****
C
MYO7A
OHC
IHC
D
OHC
IHC

Figure 5.2: Emx2-Cre Jag1 mutant reveals a role for *Jag1* in hair cell fate repression and survival of the lateral region of the auditory sensory epithelium.

(A, B) Merge showing a cross section of the wild-type ($Emx2^{+/+}$; $Jag1^{fx/fx}$) and Jag 1 mutant ($Emx2^{Cre/+}$; $Jag1^{fx/fx}$) at postnatal day 2 (P2). (A' B') Hair cells labeled with anti-parvalbumin (Blue) in the wild type and Jag1 mutant. In the Jag1 mutant two inner hair cells are present and no outer hair cells are present. (A" B") Ant-Sox2 (Red) labeling supporting cells in the wild-type and Jag1 mutant. Outer supporting cells are missing in the mutant as compared to the wild-type. (A''' B''') Anti-S100 (Green) labeling supporting cells in the wild-type and Jag1 mutant. Once again outer hair cells are missing.

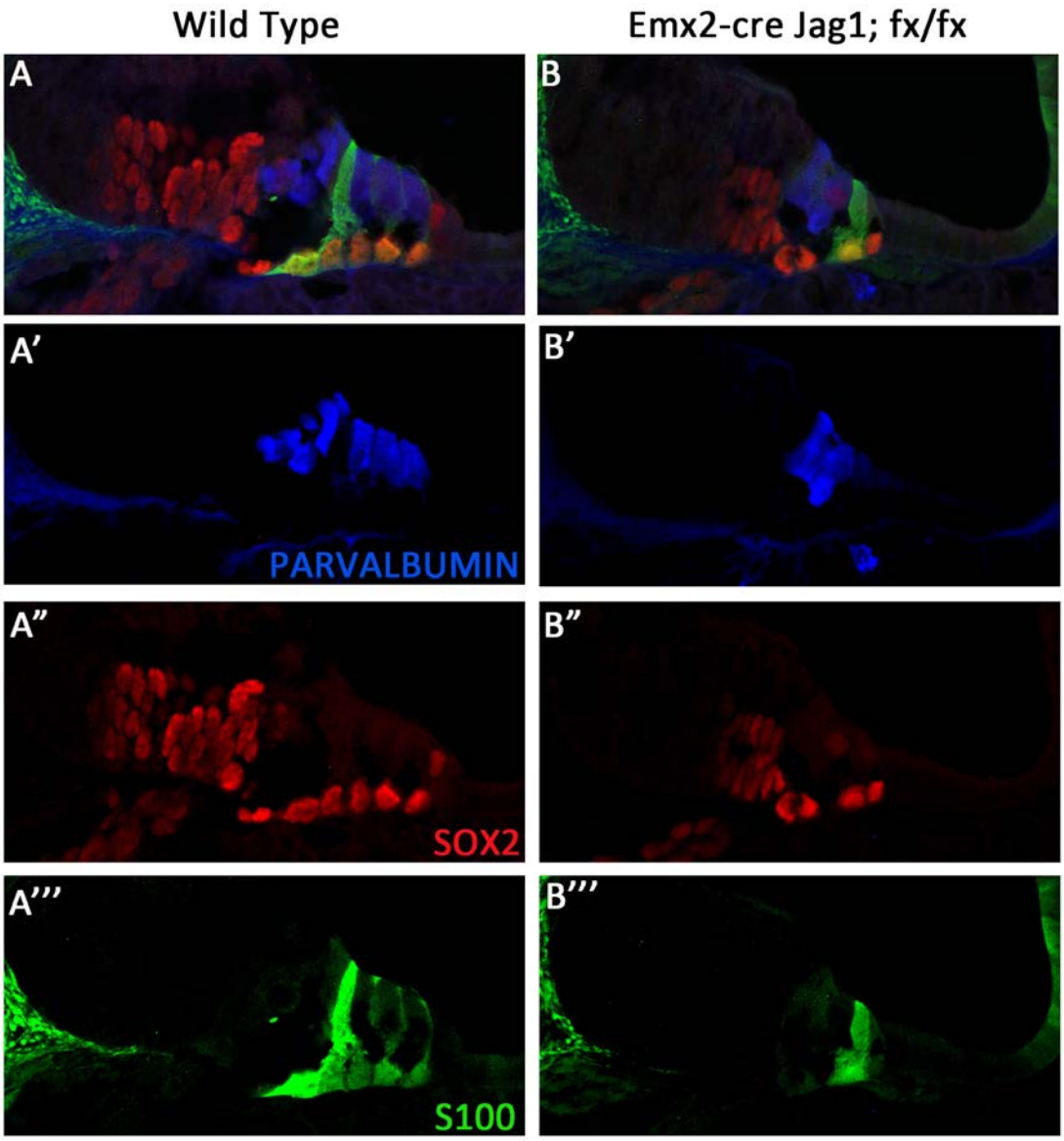
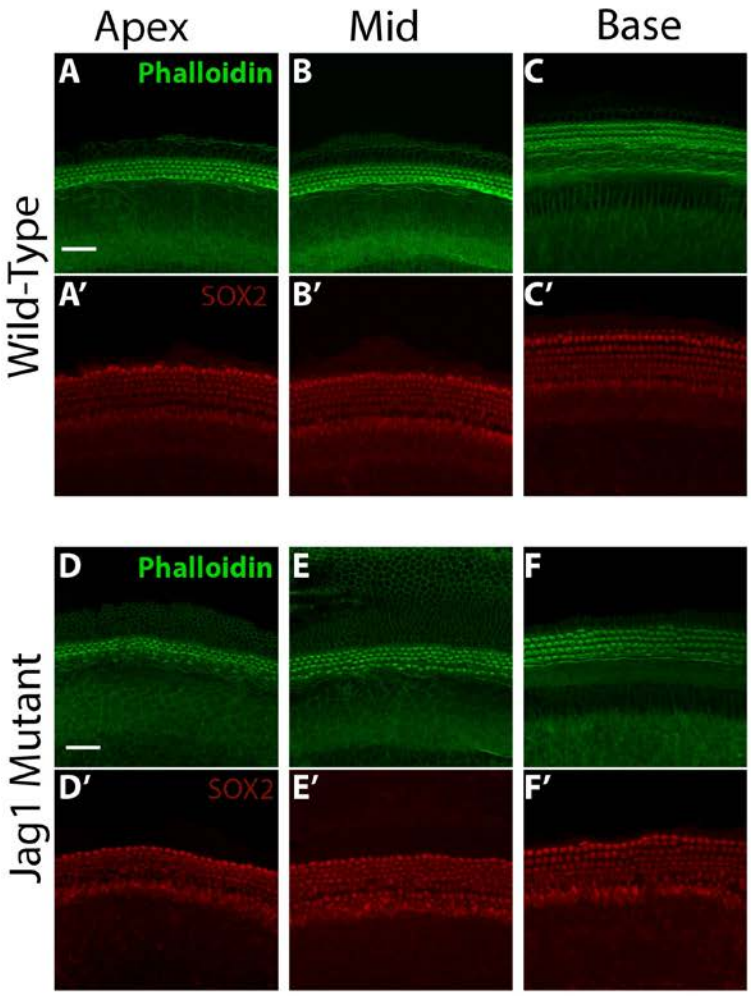
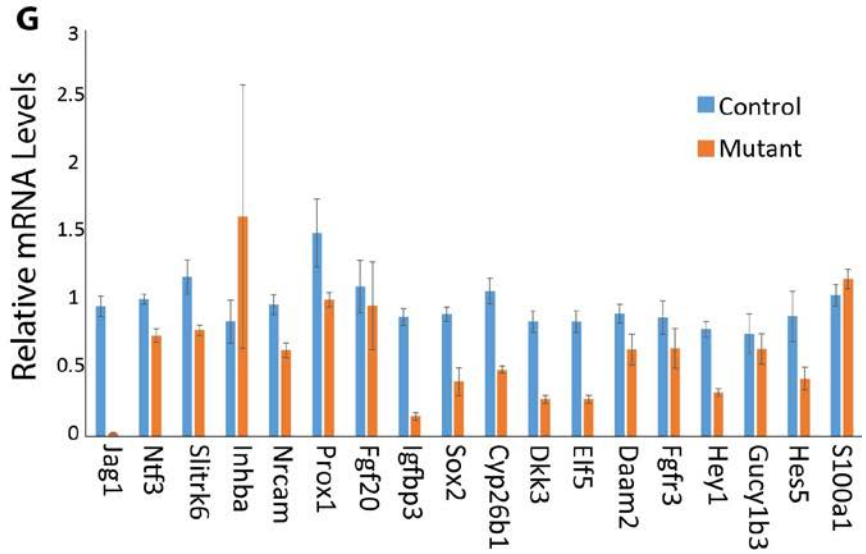

Wild Type
Emx2-cre Jag1; fx/fx
A
B
A'
B'
PARVALBUMIN
A"
B"
SOX2
A'''
B'''
S100

Figure 5.3: Jag1 is important for hair cell fate repression and supporting cell gene regulation during sensory cell differentiation

Sox2-CreER was used to delete Jag1 by activating cre with tamoxifen administration at E14.5 and E15.5. Mutant and wild type littermates were analyzed at E18.5. (A-C) Phalloidin (green) staining the stereocilia hair cell bundles showing the normal patterning of hair cells in E18.5 control ($Sox2^{+/+}$; $Jag1^{fx/fx}$) littermates. (A'-C') SOX2 staining showing the normal compliment of supporting cells in E18.5 control littermates. (D-F) Phalloidin (green) staining the stereocilia hair cell bundles showing the mild ectopic outer hair cell phenotype and the more prominent ectopic inner hair cell phenotype in E18.5 Jag1 mutant ($Sox2^{creER/+}$; $Jag1^{fx/fx}$) mice. Scale bar 20 μm. (G) Notch target gene expression in Jag1 mutant (orange bar) and wild-type cochlear epithelia (blue bar) at stage E18.5. Relative transcript levels were analyzed using RT-qPCR. Data expressed as mean ± SEM (n = 3 biological replicates).


Apex
Mid
Base
A
Phalloidin
B
C
Wild-Type
A′
SOX2
B′
C′
D
Phalloidin
E
F
Jag1 Mutant
D′
SOX2
E′
F′


G
Relative mRNA Levels
3
2.5
2
1.5
1
0.5
0
Control
Mutant
Jag1
Ntf3
Slitrk6
Inhba
Nrcam
Prox1
Fgf20
Igfbp3
Sox2
Cyp26b1
Dkk3
Elf5
Daam2
Fgfr3
Hey1
Gucy1b3
Hes5
S100a1

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CURRICULUM VITAE FOR Ph.D. CANDIDATES

The Johns Hopkins University School of Medicine

Dean P. Campbell September 1st 2015

Educational History:

Ph.D. expected	2015	Program in Biochemistry, Cellular, and Molecular Biology Mentor: Angelika Doetzlhofer, Ph.D.	Johns Hopkins University School of Medicine
B.S.	2008	Cell Biology and Biochemistry	Bucknell University

Professional Experience:

Laboratory Instructor	2015-Present	Gettysburg College
Graduate Research	August 2008-Present	Johns Hopkins University
Summer Research	June 2007 - August 2007	University of Pittsburgh

Academic and Professional Honors:

2008 Graduated Cum Laude Bucknell University
2004-2008 Dean's List Bucknell University (8 semesters)
2007 Bucknell University Phi Sigma Biology Honor society
2007 Bucknell University Mortar Board National Leadership and Service Honor Society
2006 Summer Undergraduate Research Grant Bucknell University

Conference Presentations:

Campbell D, Doetzlhofer A. Notch Signaling Plays an Instructive Role in Supporting Cell Development and is Critical for Supporting Cell Survival. Presented February 2015 at the Association for Otolaryngology Annual Meeting, Baltimore, MD (Selected for podium presentation).

Campbell D, Doetzlhofer A. The role of the Notch signaling pathway in supporting cell development. Presented February 2013 at the Association for Otolaryngology Annual Meeting, Baltimore, MD (Poster).

Campbell D, Doetzlhofer A. Function of Hey1 and HeyL in the developing mammalian cochlea. Presented November 2011. Nanosymposium on Development of Motor and Sensory Systems, Society for Neuroscience (SFN) Meeting, Washington, D.C. (talk).

Campbell D, Golden E, Barton M, Doetzlhofer A. Function of Hey1 and HeyL in the developing mammalian cochlea. Presented February 2011 at the Association for Otolaryngology Annual Meeting, Baltimore, MD (Poster)